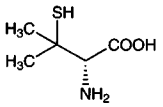
**Detector:** UV 200.5

**CHROMATOGRAM**
**Retention time:** 20.183

**KEY WORDS**
whole blood

**REFERENCE**
Gaillard,Y.; Pépin,G. Use of high-performance liquid chromatography with photodiode-array UV detection for the creation of a 600-compound library. Application to forensic toxicology, *J.Chromatogr.A*, **1997**, *763*, 149–163.

# Penicillamine




**Molecular formula:** $C_5H_{11}NO_2S$
**Molecular weight:** 149.21
**CAS Registry No.:** 52-67-5
**Merck Index:** 7214

**SAMPLE**
**Matrix:** blood
**Sample preparation:** 1 mL Plasma + 150 µL 25% trichloroacetic acid, vortex, cool on ice for 10 min, centrifuge at 6500 g for 2 min. Remove a 500 µL aliquot of the supernatant and add it to 200 µL 1% NaOH in water, add 250 µL buffer, add 1 mL 1 mM N-[p-(2-benzoxazolyl)phenyl] maleimide (Eastman) in EtOH, heat at 37° overnight, inject a 50 µL aliquot. (Buffer was 500 mM sodium citrate adjusted to pH 5.0 with perchloric acid.)

**HPLC VARIABLES**
**Column:** 300 × 3.9 10 µm µBondapak C18
**Mobile phase:** MeOH:100 µM sodium acetate 48:52
**Flow rate:** 2
**Injection volume:** 50
**Detector:** F ex 319 em 360 (cutoff filter)

**CHROMATOGRAM**
**Retention time:** 5.5
**Limit of detection:** 250 nM
**Limit of quantitation:** 1 µM

**KEY WORDS**
derivatization; plasma

**REFERENCE**
Miners,J.O.; Fearnley,I.; Smith,K.J.; Birkett,D.J.; Brooks,P.M.; Whitehouse,M.W. Analysis of D-penicillamine in plasma by fluorescence derivatisation with N-[*p*-(2-benzoxazolyl)-phenyl] maleimide and high-performance liquid chromatography, *J.Chromatogr.*, **1983**, *275*, 89–96.

**SAMPLE**
**Matrix:** blood
**Sample preparation:** Mix 1 mL plasma and 400 µL 18% (w/v) trichloroacetic acid in a 100 × 15 polypropylene tube, let stand at 0° for 5 min, centrifuge at 4° at 1700 g for 10 min, remove the supernatant as completely as possible. Suspend the precipitate in 1 mL 5% (w/v) trichloroacetic acid by stirring magnetically, centrifuge at room temperature at 2000 g for 5 min, discard the supernatant, repeat this washing step. Air-dry the precipitate then blanket it with nitrogen, add 2 mL 200 mM pH 8.0 Tris buffer, pass nitrogen over the mixture for 1 h, add 100 µL 250 mM EDTA, add 50 µL octanol, add 100 mg solid sodium borohydride, remove the

hydrogen line, allow hydrogen to vent through a pin-hole in the cap, stir slowly, after 10 min cool in ice and slowly add 1 mL ice-cold 2 M perchloric acid, stir briefly, centrifuge an aliquot at room temperature at 8000 g for 30 s, remove 200 μL of the supernatant and add it to 10 μL 1 mM L-cysteine in water, inject an aliquot.

### HPLC VARIABLES

**Column:** 100 × 4.6 3 μm C18 "Short One" (Rainin)
**Mobile phase:** MeCN:buffer 6:94 (Buffer was 100 mM pH 3.0 monochloroacetic acid/NaOH containing 1 g/L heptanesulfonic acid.) (Rigorously degas mobile phase by refluxing, negative-pressure filtration, and passing helium through it for 3 h before starting the assay.)
**Flow rate:** 0.6
**Injection volume:** 20-50
**Detector:** E, Bioanalytical Systems BAS LC-4B/19, BAS TL-6A Au/Hg working electrode + 150 mV, glassy carbon auxiliary electrode, Ag/AgCl reference electrode

### CHROMATOGRAM

**Retention time:** 4.66
**Limit of detection:** 1.2 μM

### KEY WORDS

plasma

### REFERENCE

Joyce,D.A.; Wade,D.N. Assay for D-penicillamine-protein conjugate in human plasma utilising chemical reduction followed by high-performance liquid chromatography with gold/mercury electrochemical detection, *J.Chromatogr.*, **1988**, *430*, 319–327.

## SAMPLE

**Matrix:** blood
**Sample preparation:** 1 Volume plasma + 0.4 volume 10 mM monobromobimane (Calbiochem; Molecular Probes, Eugene OR) + 0.01 volume 1 M acetic acid + 2.5 volume MeCN, mix, centrifuge at 4° at 1000 g for 10 min, filter (5 μm) the supernatant, inject a 100 μL aliquot.

### HPLC VARIABLES

**Column:** μBondapak C18 radial compression
**Mobile phase:** MeOH:water:glacial acetic acid 22:77.75:0.25, pH adjusted to 3.9 with NaOH. (After each injection wash column with MeOH at 4 mL/min for 10 min.)
**Flow rate:** 1.5
**Injection volume:** 100
**Detector:** F ex 365 em 418

### CHROMATOGRAM

**Retention time:** 13.60
**Internal standard:** d-penicillamine
**Limit of detection:** 10 nM

### OTHER SUBSTANCES

**Extracted:** cysteine, glutathione, homocysteine

### KEY WORDS

derivatization; plasma; penicillamine is IS

### REFERENCE

Velury,S.; Howell,S.B. Measurement of plasma thiols after derivatization with monobromobimane, *J.Chromatogr.*, **1988**, *424*, 141–146.

## SAMPLE

**Matrix:** blood
**Sample preparation:** 50 μL Plasma + 10 μL 25 mM monobromobimane in MeCN, let stand for 5 min at room temperature, add 20 μL 20% perchloric acid.

### HPLC VARIABLES

**Column:** 150 × 4.6 7 μm Nucleosil RP-18
**Mobile phase:** Gradient. A was MeCN. B was 1% aqueous acetic acid containing 1 g/L octanesulfonic acid. A:B from 5:95 to 8:92 over 2 min, to 10:90 over 13 min (Waters convex), to 30:70 over 20 min (Waters convex), maintain at 30:70 for 6 min, re-equilibrate at initial conditions for 9 min.
**Flow rate:** 1.4
**Detector:** F (wavelengths not given)

### CHROMATOGRAM

**Internal standard:** penicillamine

### OTHER SUBSTANCES

**Extracted:** mesna

### KEY WORDS

plasma; derivatization; penicillamine is IS

### REFERENCE

Stofer-Vogel,B.; Cerny,T.; Borner,M.; Lauterburg,B.H. Oral bioavailability of mesna tablets, *Cancer Chemother.Pharmacol.*, **1993**, *32*, 78–81.

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### SAMPLE

**Matrix:** blood, cells
**Sample preparation:** Blood. Mix 9 mL whole blood with 1 mL 3.8% sodium citrate, centrifuge at 4° at 150 g for 15 min, wash the erythrocytes three times with isotonic saline. Suspend 100 μL erythrocytes in 700 μL 6 mM EDTA, mix gently for 1 min, add 200 μL 25% metaphosphoric acid, mix for 10 min, centrifuge at 5000 g for 15 min, filter (0.45 μm) the supernatant, inject a 10 μL aliquot of the filtrate. Cells. Wash 500 mg (wet weight) E. coli cells with water, add 2 mL 5% metaphosphoric acid, sonicate, centrifuge at 4° at 15000 g for 15 min, filter (0.45 μm) the supernatant, inject an aliquot of the filtrate.

### HPLC VARIABLES

**Column:** 250 × 4.6 Fine Sil C18-10 (Japan Spectroscopic)
**Mobile phase:** 33 mM $KH_2PO_4$ adjusted to pH 2.2 with phosphoric acid
**Flow rate:** 1
**Injection volume:** 10
**Detector:** UV 344 following post-column reaction. The column effluent mixed with the 1.5 mM 6,6'-dithiodinicotinic acid in 200 mM pH 7.0 sodium phosphate buffer pumped at 1 mL/min and the mixture flowed through a 60 cm × 0.5 mm ID stainless steel coil to the detector.

### CHROMATOGRAM

**Retention time:** 8.2
**Limit of detection:** 0.1 nmole

### OTHER SUBSTANCES

**Extracted:** cysteamine, cysteine, glutathione, homocysteine

### KEY WORDS

post-column reaction; whole blood; erythrocytes

### REFERENCE

Nishiyama,J.; Kuninori,T. Assay of biological thiols by a combination of high-performance liquid chromatography and postcolumn reaction with 6,6'-dithiodinicotinic acid, *Anal.Biochem.*, **1984**, *138*, 95–98.

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### SAMPLE

**Matrix:** blood, sea water, urine
**Sample preparation:** Blood. Centrifuge blood at 550 g for 30 min. Mix the clear solution with MeOH and centrifuge at 500 g for 30 min. Filter (0.22 mm) and centrifuge at 2500 g. Dilute the clear liquid with an equal amount of mobile phase. Centrifuge 2500 g for 5 min inject an

aliquot of the supernatant. Sea water, urine. Filter (0.22 mm), dilute with an equal amount of mobile phase, store at -5°, inject an aliquot.

### HPLC VARIABLES

**Column:** 250 × 4.6 5 μm Zorbax ODS
**Mobile phase:** MeOH:50 mM pH 2.2 trichloroacetic acid 1:99
**Column temperature:** 25
**Flow rate:** 0.3
**Injection volume:** 50
**Detector:** E, PAR-174, 1.0 mm tungsten wire electrode at -0.3 V, Ag/AgCl reference electrode, 1.0 mm dia. Pt wire auxiliary electrode (construction details for cell in paper) following post-column reaction. The column effluent mixed with 100 mM pH 3.1 phosphate buffer containing 25 μM or 25 mM $Hg^{2+}$ (both quantities in paper) pumped at 1.0 mL/min and the mixture flowed to the detector.

### CHROMATOGRAM

**Retention time:** 16.0
**Limit of detection:** 1 ng

### OTHER SUBSTANCES

**Extracted:** cysteine, glutathione, homocysteine, mercaptopropionic acid, thiourea

### KEY WORDS

plasma

### REFERENCE

Hidayat,A.; Hibbert,D.B.; Alexander,P.W. Amperometric detection of organic thiols at a tungsten wire electrode following their separation by liquid chromatography, *J.Chromatogr.B*, **1997**, *693*, 139–146.

### SAMPLE

**Matrix:** blood, tissue
**Sample preparation:** Plasma. 1 mL Plasma + 1 mL ice-cold 2 M perchloric acid containing 4 mM EDTA, vortex, centrifuge at 4° at 4000 g for 10 min. Neutralize an aliquot of the supernatant with cold 2 M LiOH solution, adjust to 10% with pH 8.4 borate buffer. Remove a 100 μL aliquot and add it to 100 μL 50 mM N-acetylcysteine solution, 100 μL reagent solution, and 700 μL pH 8.4 borate buffer, vortex for a few s, let stand at room temperature for 1 h, add an equal volume of the mobile phase, inject a 10 μL aliquot. Tissue. Powder tissue at low temperature. Add 4 volumes ice-cold 2 M perchloric acid containing 25 mM EDTA to 0.1-0.5 g powdered tissue, vortex quickly, homogenize (Brinkman). Neutralize an aliquot with cold 2 M LiOH solution, adjust to 1% (brain, lung, liver, kidney, testes, small intestine) or 2.5% (aorta, heart, spleen) with pH 8.4 borate buffer. Remove a 100 μL aliquot and add it to 100 μL 50 mM N-acetylcysteine solution, 100 μL reagent solution, and 700 μL pH 8.4 borate buffer, vortex for a few s, let stand at room temperature for 1 h, add an equal volume of the mobile phase, inject a 10 μL aliquot. (Prepare reagent, 2-(4-N-maleimidephenyl)-6-methylbenzothiazole, as follows. Recrystallize 2-(4-aminophenyl)-6-methylbenzothiazole from chloroform before use. Add 500 mg maleic anhydride in 2 mL chloroform dropwise to 1.2 g 2-(4-aminophenyl)-6-methylbenzothiazole in 10 mL DMF, stir at room temperature for 2 h, filter, wash with 30 mL chloroform, recrystallize from DMF to give 2-(4-N-phenylmaleamic acid)-6-methylbenzothiazole as yellow crystals (mp 242°). Reflux 2 g 2-(4-N-phenylmaleamic acid)-6-methylbenzothiazole, 100 mg anhydrous sodium acetate, and 25 mL acetic anhydride for 2 h, cool on ice, filter, wash the solid with water. Neutralize the filtrate with cold 10% NaOH, extract with chloroform. Dry the organic layer over anhydrous magnesium sulfate and evaporate it to dryness under reduced pressure. Combine this product with the solid obtained earlier and recrystallize from isopropanol to give 2-(4-N-maleimidephenyl)-6-methylbenzothiazole as yellow needles (mp 254-6°). Prepare the reagent solution by dissolving 50 μmoles of this compound in 10 mL DMF and diluting 25-fold with MeCN.)

### HPLC VARIABLES

**Column:** 250 × 4.6 5 μm Ultrasphere ODS
**Mobile phase:** MeCN:buffer 35:65, pH 4.5 (Buffer was 10 mM $KH_2PO_4$ containing 0.1% sodium hexanesulfonate.)
**Flow rate:** 1.5
**Injection volume:** 10

**Detector:** F ex 320 em 405

**CHROMATOGRAM**
**Retention time:** 17
**Internal standard:** N-acetylcysteine (7)
**Limit of detection:** 20 fmole

**OTHER SUBSTANCES**
**Extracted:** N-acetylpenicillamine, coenzyme A, cysteine, glutathione, homocysteine

**KEY WORDS**
derivatization; plasma; rat; aorta; heart; lung; liver; kidney; testes; spleen; brain; small intestine

**REFERENCE**
Haj-Yehia,A.I.; Benet,L.Z. Determination of aliphatic thiols by fluorometric high-performance liquid chromatography after precolumn derivatization with 2-(4-N-maleimidophenyl)-6-methylbenzothiazole,*Pharm.Res.*, **1995**, *12*, 155–160.

**SAMPLE**
**Matrix:** bulk
**Sample preparation:** Dissolve 5 mg amino acids in 10 mL MeCN:water:triethylamine 50:50:0.55. Remove a 50 μL aliquot and add it to 50 μL 0.66% 2,3,4,6-tetra-O-benzoyl-β-D-glucopyranosyl isothiocyanate (Fluka) in MeCN, shake mechanically for 30 min, add 10 μL 0.26% ethanolamine in MeCN, shake for 10 min, make up to 1 mL with MeCN, inject a 10 μL aliquot.

**HPLC VARIABLES**
**Column:** 25 × 4 (sic) 5 μm LiChrospher 100 RP-18
**Mobile phase:** MeOH:water:67 mM pH 7.0 phosphate buffer 70:25:5
**Flow rate:** 0.45
**Injection volume:** 10
**Detector:** UV 231

**CHROMATOGRAM**
**Retention time:** k' 7.37 (L), 10.05 (D)

**OTHER SUBSTANCES**
**Simultaneous:** amino acids

**KEY WORDS**
derivatization; chiral

**REFERENCE**
Lobell,M.; Schneider,M.P. 2,3,4,6-Tetra-O-benzoyl-β-D-glucopyranosyl isothiocyanate: an efficient reagent for the determination of enantiomeric purities of amino acids, β-adrenergic blockers and alkyloxiranes by high-performance liquid chromatography using standard reversed-phase columns, *J.Chromatogr.*, **1993**, *633*, 287–294.

**SAMPLE**
**Matrix:** formulations
**Sample preparation:** Dissolve about 1 mg of the contents of a capsule in 10 mL water and dilute with MeCN to a penicillamine concentration of 8 mM. 50 μL Solution + 200 μL buffer + 150 μL reagent, heat at 60° for 30 min, cool, inject a 10 μL aliquot. (Buffer was prepared by adjusting the pH of a solution containing 100 mM boric acid and 100 mM KCl to 8.5 with 100 mM sodium carbonate. Reagent was 200 μM N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM) in MeCN. Synthesis of N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide is as follows. Add 8.8 g aluminum trichloride to 12.50 g 3-dimethylaminophenol in 185 mL chloroform and 84 g triethyl orthoformate, mix at room temperature for 10 min, when the exothermic reaction ceases add 50 mL 10% HCl, stir to hydrolyze the acetal, neutralize with 10% NaOH, filter through a short column of Celite, wash through with chloroform, wash the filtrate with saturated aqueous NaCl, dry over magnesium sulfate, concentrate under reduced pressure, recrystallize from chloroform to give 4-(dimethylam-

ino)salicylaldehyde (mp 78-79°). Add 400 mg KOH in 3 mL EtOH to a solution of 1 g 4-(dimethylamino)salicylaldehyde and 1.3 g (?) 4-nitrobenzylbromide in 12 mL EtOH, reflux for 7 h, cool, filter to recover the crystals, wash with water, dry under vacuum, recrystallize from EtOH to give 4-dimethylamino-2-(4-nitrobenzyloxy)benzaldehyde (mp 179-180°). Add a solution of 900 mg 4-dimethylamino-2-(4-nitrobenzyloxy)benzaldehyde in 6 mL DMF to a sodium methoxide solution (prepared from 69 mg sodium in 1 mL MeOH), reflux for 20 min, add 1 mL MeOH, filter the crystals, recrystallize from EtOH to give 6-dimethylamino-2-(4-nitrophenyl)benzofuran as red needles (mp 209.5-210.5°). Reflux 1 g 6-dimethylamino-2-(4-nitrophenyl)benzofuran in 20 mL benzene (Caution! Benzene is a carcinogen!) and 18 mL MeOH containing 80 mg active carbon and a catalytic amount of ferric chloride hexahydrate for 10 min, add 2.30 g 98% hydrazine hydrate (Caution! Hydrazine hydrate is a carcinogen!) dropwise, reflux for 7 h, filter, concentrate the filtrate, recrystallize from cyclohexane to give 6-dimethylamino-2-(4-aminophenyl)benzofuran as orange needles (mp 198.5-200°). Stir 605 mg 6-dimethylamino-2-(4-aminophenyl)benzofuran and 230 mg maleic anhydride in 5 mL chloroform at room temperature for 3 h, filter the crystals, wash with a small amount of chloroform, recrystallize from EtOH to obtain N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleamic acid (mp 219.5-221°). Reflux a mixture of 1.17 g N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleamic acid and 30 mg sodium acetate in 18 mL acetic anhydride, cool in an ice bath, collect the crystals of product, wash with water. Neutralize the filtrate with 20% NaOH, extract twice with 30 mL portions of chloroform, wash the organic layers with saturated aqueous NaCl, dry over anhydrous magnesium sulfate, evaporate to give more product. Combine the products and recrystallize them from acetone to give N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide as reddish purple crystals (mp 203-204°) (Bull.Chem.Soc.Jpn. 1985, 58, 2192).)

## HPLC VARIABLES

**Column:** 250 × 4.6 5 μm Sumichiral OA-2500S Pirkle-type (Sumika Chemical Analysis Service)
**Mobile phase:** MeOH:water 75:25 containing 150 mM ammonium acetate and 50 mM tetra-n-butylammonium bromide
**Flow rate:** 1
**Injection volume:** 10
**Detector:** F ex 360 em 455

## CHROMATOGRAM

**Retention time:** 27 (D), 31 (L) (Each enantiomer gives 2 peaks, the later peaks are used for quantitation.)
**Limit of detection:** 350 fmole (L), 290 fmole (D)

## KEY WORDS

capsules; chiral; derivatization

## REFERENCE

Nakashima,K.; Ishimaru,T.; Kuroda,N.; Akiyama,S. High-performance liquid chromatographic separation of penicillamine enantiomers labelled with *N*-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide on a chiral stationary phase, *Biomed.Chromatogr.*, **1995**, *9*, 90–93.

## SAMPLE

**Matrix:** formulations
**Sample preparation:** Dissolve about 1 mg of the contents of a capsule in 10 mL water and dilute with MeCN to a penicillamine concentration of 8 mM. 50 μL Solution + 200 μL buffer + 150 μL reagent, heat at 60° for 30 min, cool, inject a 10 μL aliquot. (Buffer was prepared by adjusting the pH of a solution containing 100 mM boric acid and 100 mM KCl to 8.5 with 100 mM sodium carbonate. Reagent was 200 μM N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide (DBPM) in MeCN. Synthesis of N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide is as follows. Add 8.8 g aluminum trichloride to 12.50 g 3-dimethylaminophenol in 185 mL chloroform and 84 g triethyl orthoformate, mix at room temperature for 10 min, when the exothermic reaction ceases add 50 mL 10% HCl, stir to hydrolyze the acetal, neutralize with 10% NaOH, filter through a short column of Celite, wash through with chloroform, wash the filtrate with saturated aqueous NaCl, dry over magnesium sulfate, concentrate under reduced pressure, recrystallize from chloroform to give 4-(dimethylamino)salicylaldehyde (mp 78-79°). Add 400 mg KOH in 3 mL EtOH to a solution of 1 g 4-(dimethylamino)salicylaldehyde and 1.3 g (?) 4-nitrobenzylbromide in 12 mL EtOH, reflux for 7 h, cool, filter to recover the crystals, wash with water, dry under vacuum, recrystallize from

EtOH to give 4-dimethylamino-2-(4-nitrobenzyloxy)benzaldehyde (mp 179-180°). Add a solution of 900 mg 4-dimethylamino-2-(4-nitrobenzyloxy)benzaldehyde in 6 mL DMF to a sodium methoxide solution (prepared from 69 mg sodium in 1 mL MeOH), reflux for 20 min, add 1 mL MeOH, filter the crystals, recrystallize from EtOH to give 6-dimethylamino-2-(4-nitrophenyl)benzofuran as red needles (mp 209.5-210.5°). Reflux 1 g 6-dimethylamino-2-(4-nitrophenyl)benzofuran in 20 mL benzene (Caution! Benzene is a carcinogen!) and 18 mL MeOH containing 80 mg active carbon and a catalytic amount of ferric chloride hexahydrate for 10 min, add 2.30 g 98% hydrazine hydrate (Caution! Hydrazine hydrate is a carcinogen!) dropwise, reflux for 7 h, filter, concentrate the filtrate, recrystallize from cyclohexane to give 6-dimethylamino-2-(4-aminophenyl)benzofuran as orange needles (mp 198.5-200°). Stir 605 mg 6-dimethylamino-2-(4-aminophenyl)benzofuran and 230 mg maleic anhydride in 5 mL chloroform at room temperature for 3 h, filter the crystals, wash with a small amount of chloroform, recrystallize from EtOH to obtain N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleamic acid (mp 219.5-221°). Reflux a mixture of 1.17 g N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleamic acid and 30 mg sodium acetate in 18 mL acetic anhydride, cool in an ice bath, collect the crystals of product, wash with water. Neutralize the filtrate with 20% NaOH, extract twice with 30 mL portions of chloroform, wash the organic layers with saturated aqueous NaCl, dry over anhydrous magnesium sulfate, evaporate to give more product. Combine the products and recrystallize them from acetone to give N-[4-(6-dimethylamino-2-benzofuranyl)phenyl]maleimide as reddish purple crystals (mp 203-204°) (Bull.Chem.Soc.Jpn. 1985, 58, 2192).)

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**HPLC VARIABLES**

**Column:** 250 × 4.6 5 μm Sumichiral OA-2500S Pirkle-type (Sumika Chemical Analysis Service)
**Mobile phase:** MeOH:water 75:25 containing 150 mM ammonium acetate and 50 mM tetra-n-butylammonium bromide
**Flow rate:** 1
**Injection volume:** 10
**Detector:** F ex 360 em 455

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**CHROMATOGRAM**

**Retention time:** 27 (D), 31 (L) (Each enantiomer gives 2 peaks, the later peaks are used for quantitation.)
**Limit of detection:** 350 fmole (L), 290 fmole (D)

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**KEY WORDS**

capsules; chiral; derivatization

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**REFERENCE**

Reichelova,V.; Liliemark,J.; Albertioni,F. Structure-activity relationships of 2-chloro-2'-*arabino*-fluoro-2'-deoxyadenosine and related analogues: Protein binding, lipophilicity, and retention in reversed-phase LC, *J.Liq.Chromatogr.*, **1995**, *18*, 1123–1135.

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**SAMPLE**

**Matrix:** solutions
**Sample preparation:** Add 1 mL 50 μg/mL N-(4-anilinophenyl)maleimide in 33 mM pH 6.85 phosphate buffer to 0.1-4 μg thiol, let stand at 0° for 90 min, wash twice with 2 mL portions of ether, heat the aqueous phase to 50° for 20 min, inject an aliquot. (Prepare N-(4-anilinophenyl)maleimide as follows. Add dropwise 1.1 g maleic anhydride in 10 mL chloroform to 1 g N-phenylphenylenediamine (4-aminodiphenylamine) stirred in 10 mL chloroform at 0°, filter, dry to give N-(4-anilinophenyl)maleamic acid. Heat 100 mg N-(4-anilinophenyl)maleamic acid and 25 mg sodium acetate in 400 μL acetic anhydride on a water bath for 2 h, cool, pour into ice-water, filter, recrystallize from ethyl acetate/hexane to give N-(4-anilinophenyl)maleimide as yellow needles (mp 135-6°).)

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**HPLC VARIABLES**

**Column:** 305 × 6.3 μBondapak C18
**Mobile phase:** MeCN:0.5% pH 3.0 $(NH_4)H_2PO_4$ 4:7
**Flow rate:** 1
**Injection volume:** 10
**Detector:** E, Yanagimoto model VMD-101, glassy carbon electrode +1.0 V, Ag/AgCl reference electrode

**CHROMATOGRAM**
**Retention time:** 12

**OTHER SUBSTANCES**
**Simultaneous:** N-acetyl-L-cysteine, L-cysteine, glutathione

**KEY WORDS**
derivatization

**REFERENCE**
Shimada,K.; Tanaka,M.; Nambara,T. Sensitive derivatization reagents for thiol compounds in high-performance liquid chromatography with electrochemical detection, *Anal.Chim.Acta*, **1983**, *147*, 375–380.

**SAMPLE**
**Matrix:** solutions
**Sample preparation:** Add 1.05-3 equivalents 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate to 10 mL of a 100 μM solution of the thiol in MeCN:water 50:50 containing 1-3 equivalents triethylamine, vortex briefly, let stand at room temperature for 30 min, dilute with mobile phase, inject a 20 μL aliquot.

**HPLC VARIABLES**
**Column:** 150 × 4.6 5 μm TSKgel ODS-80TM (Tosoh)
**Mobile phase:** MeCN:10 mM pH 2.8 potassium phosphate buffer 60:40
**Column temperature:** 40
**Flow rate:** 1
**Injection volume:** 20
**Detector:** UV 250

**CHROMATOGRAM**
**Retention time:** 3.99 (L), 5.24 (D)

**OTHER SUBSTANCES**
**Simultaneous:** cysteine, homocysteine

**KEY WORDS**
derivatization; chiral

**REFERENCE**
Ito,S.; Ota,A.; Yamamoto,K.; Kawashima,Y. Resolution of the enantiomers of thiol compounds by reversed-phase liquid chromatography using chiral derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate, *J.Chromatogr.*, **1992**, *626*, 187–196.

**SAMPLE**
**Matrix:** solutions
**Sample preparation:** Mix 20 μL of a 30 μM solution in 2 mM disodium EDTA containing 3% triethylamine with 10 μL 12 mM R-(-)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole in MeCN, let stand for 40 min, inject a 10 μL aliquot. (Synthesis of (R)-(-)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole, (R)-(-)-NBD-PyNCS, is as follows. Cool a solution of 16.4 g (S)-(-)-1-benzyl-3-pyrrolidinol in 164 mL pyridine to +5°, add 19.35 g p-toluenesulfonyl chloride, stir at +10° for 48 h, evaporate to dryness, chromatograph using dichloromethane:acetone 95:5 to obtain (3S)-3-[(4-tolylsulfonyl)oxy]-1-(phenylmethyl)pyrrolidine (mp 68°). Heat a solution of (3S)-3-[(4-tolylsulfonyl)oxy]-1-(phenylmethyl)pyrrolidine in 200 mL anhydrous DMF to 65°, add 33.5 g sodium azide (Caution! Sodium azide is highly toxic!), stir at 60° for 7 h, filter, evaporate the filtrate to dryness under reduced pressure, dissolve the residue in ethyl acetate, wash twice with water, dry over anhydrous magnesium sulfate, evaporate to obtain (3R)-3-azido-1-(phenylmethyl)pyrrolidine as an oil. Add 3.5 g 10% palladium on carbon under nitrogen to a solution of 7.05 g (3R)-3-azido-1-(phenylmethyl)pyrrolidine in 34.8 mL 1 M HCl in water and 245 mL EtOH, hydrogenate at atmospheric pressure for 30 min, add 3.5 g catalyst, hydrogenate for 2 h, filter, add 34.8 mL 1 M HCl to the filtrate, evaporate to dryness under reduced pressure, take up the residue in 70 mL EtOH, filter, evaporate the filtrate to dryness under reduced pressure, repeat this operation twice, crystallize with the

minimum amount of EtOH to obtain (3R)-3-aminopyrrolidine dihydrochloride (J. Med. Chem. 1992, 35, 4205). 3R-(+)-aminopyrrolidine is also reported to be available from Tokyo Kasei. Add 100 mg 4-fluoro-7-nitro-2,1,3-benzoxadiazole in 20 mL MeCN dropwise to a stirred solution of 200 mg 3R-(+)-aminopyrrolidine in 20 mL MeCN at 0-10°, stir at room temperature for 30 min, remove the MeCN by evaporation under reduced pressure, dissolve the residue in 50 mL water, extract 4 times with 80 mL portions of ethyl acetate. Combine the organic layers and wash them with 20 mL water, dry over anhydrous sodium sulfate, evaporate to dryness under reduced pressure, recrystallize from hexane to obtain (R)-(-)-4-(3-aminopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole as dark red crystals (mp 178-181°) (Analyst 1992, 117, 727). Add 100 μL thiophosgene in 10 mL benzene (Caution! Benzene is a carcinogen!) to 100 mg (R)-(-)-4-(3-aminopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole in 100 mL acetone, reflux for 1 h, remove the solvent by evaporation under reduced pressure, suspend the residue in 100 mL water, extract 4 times with 25 mL portions of benzene. Combine the extracts and wash them with 20 mL water, dry over anhydrous sodium sulfate, evaporate to dryness under reduced pressure, recrystallize from hexane:benzene 1:2 to obtain (R)-(-)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-nitro-2,1,3-benzoxadiazole as red crystals (mp 165-170°) (Analyst 1995, 120, 385).)

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**HPLC VARIABLES**
**Column:** 150 × 4.6 5 μm Ultron VX-ODS (Shinwa, Kyoto)
**Mobile phase:** MeCN:water:trifluoroacetic acid 35:65:0.1
**Column temperature:** 40
**Flow rate:** 1
**Injection volume:** 10
**Detector:** F ex 455 em 568

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**CHROMATOGRAM**
**Retention time:** 20, 23 (enantiomers)

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**KEY WORDS**
derivatization; chiral

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**REFERENCE**
Jin,D.; Takehana,K.; Toyo'oka,T. Chiral separation of racemic thiols based on diastereomer formation with a fluorescent chiral tagging reagent by reversed-phase liquid chromatography, *Anal.Sci.*, **1997**, *13*, 113–115.

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**SAMPLE**
**Matrix:** solutions
**Sample preparation:** Mix a 200 μM solution in buffer with three volumes of a 400 μM solution of 5,5'-dithio-(bis-2-nitrobenzoic acid) in buffer, let stand at room temperature for 30 min, inject a 75 μL aliquot. (Buffer was 125 mM $NaH_2PO_4$ containing 154 mM NaCl, pH adjusted to 7.4 with NaOH.)

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**HPLC VARIABLES**
**Column:** 250 × 4.6 Hypersil ODS1
**Mobile phase:** Gradient. MeCN:buffer 0:100 for 20 min, to 17.5:82.5 over 40 min. (Buffer was 125 mM $NaH_2PO_4$ containing 154 mM NaCl, pH adjusted to 7.4 with NaOH.)
**Flow rate:** 0.25 for 20 min, to 1 over 40 min
**Injection volume:** 75
**Detector:** UV 357

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**CHROMATOGRAM**
**Retention time:** 45

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**OTHER SUBSTANCES**
**Simultaneous:** N-acetylcysteine, N-acetylpenicillamine, captopril, cysteine, glutathione, thiomalic acid

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**KEY WORDS**
derivatization

### REFERENCE

Russell,J.; McKeown,J.A.; Hensman,C.; Smith,W.E.; Reglinski,J. HPLC determination of biologically active thiols using pre-column derivatization with 5,5'-dithio-(bis-2-nitrobenzoic acid), *J.Pharm.Biomed.Anal.*, **1997**, *15*, 1757–1763.

### SAMPLE

**Matrix:** tissue

**Sample preparation:** Freeze tissue in liquid nitrogen and pulverize. Homogenize 50-100 mg tissue in 1 mL MeCN:20 mM EDTA 30:70, centrifuge at 4° at 4000 g for 5 min, adjust to 1-2.5% w/v with pH 8.4 borate buffer, keep on ice. 100 μL Sample + 100 μL 0.05 mM N-acetylcysteine + 100 μL 0.25 mM reagent in MeCN:DMF 95:5 + 700 μL pH 8.4 borate buffer, vortex for a few s, let stand for 1 h at room temperature, dilute with an equal volume of mobile phase, inject a 10 μL aliquot. (Reagent was 2-(4-maleimidophenyl)-6-methoxybenzofuran, a partial synthesis is given in the paper.)

### HPLC VARIABLES

**Column:** 250 × 4.6 5 μm Ultrasphere-ODS

**Mobile phase:** MeCN:buffer 35:65 adjusted to pH 4.5 (Buffer was 10 mM $KH_2PO_4$ containing 0.1% sodium hexanesulfonate.)

**Flow rate:** 1.5

**Injection volume:** 10

**Detector:** F ex 310 em 390

### CHROMATOGRAM

**Retention time:** 10

**Internal standard:** N-acetylcysteine (8)

**Limit of detection:** 75 fmole

### OTHER SUBSTANCES

**Extracted:** glutathione, homomocysteine, acetylpenicillamine

### KEY WORDS

rat; heart; lung; liver; kidney; testes; spleen; derivatization

### REFERENCE

Haj-Yehia,A.I.; Benet,L.Z. 2-(4-N-Maleimidophenyl)-6-methoxybenzofuran: a superior derivatizing agent for fluorimetric determination of aliphatic thiols by high-performance liquid chromatography, *J.Chromatogr.B*, **1995**, *666*, 45–53.

### SAMPLE

**Matrix:** urine

**Sample preparation:** For each 1 mL urine add 1-2 mg EDTA and 5 μg homocysteine, inject a 20 μL aliquot.

### HPLC VARIABLES

**Guard column:** 45 × 4.6 5 μm ODS Hypersil

**Column:** 100 × 4.6 5 μm ODS Hypersil

**Mobile phase:** 1 g/L pH 4 Heptanesulfonic acid in water containing 150 mg/L sodium EDTA

**Flow rate:** 1

**Injection volume:** 20

**Detector:** UV 412 following post-column reaction. The column effluent mixed with the reagent pumped at 0.5 mL/min and the mixture flowed through a 150 × 2 column filled with 40 μm glass beads to the detector. (Prepare reagent by dissolving 200 mg 5,5'-dithiobis(2-nitrobenzoic acid) and 10 g tripotassium citrate in 100 mL 250 mM pH 7.4 phosphate buffer, dilute 10-fold with water immediately before use.)

### CHROMATOGRAM

**Retention time:** 1.3

**Internal standard:** homocysteine (0.8)

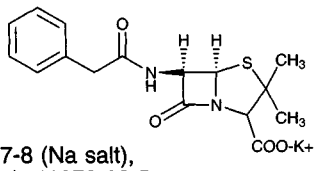
**Limit of quantitation:** 10 ng

**OTHER SUBSTANCES**
**Extracted:** cysteine

**KEY WORDS**
post-column reaction

**REFERENCE**
Beales,D.; Finch,R.; McLean,A.E.M.; Smith,M.; Wilson,I.D. Determination of penicillamine and other thiols by combined high-performance liquid chromatography and post-column reaction with Ellman's reagent: application to human urine, *J.Chromatogr.*, **1981**, *226*, 498–503.

# Penicillin G




**Molecular formula:** $C_{16}H_{18}N_2O_4S$
**Molecular weight:** 334.40
**CAS Registry No.:** 113-98-4 (potasium salt), 61-33-6 (free acid), 69-57-8 (Na salt), 751-84-8 (penicillin G benethamine), 1538-09-6 (penicillin G benzathine), 41372-02-5 (penicillin G benzathine tetrahydrate), 1538-11-0 (penicillin G benzhydrylamine), 973-53-5 (Ca salt), 3344-16-9 (penicillin G hydrabamine), 6130-64-9 (penicillin G procaine monohydrate), 54-35-3 (penicillin G procaine)
**Merck Index:** 7225

**SAMPLE**
**Matrix:** bile, blood, urine
**Sample preparation:** Serum. 0.5 mL serum + 0.5 mL MeCN mix in 7 mL tube on vortex mixer; shake by rotation (20 rpm) 10 min; centrifuge 10 min 1000 g; transfer supernatant to another tube, add 7 aliquots dichloromethane; equilibrate 10 min; shake by rotation (20 rpm) 10 min; centrifuge 10 min 1000 g; inject aliquot of upper aqueous layer. Urine. Centrifuge urine and dilute 1:20. Bile. Centrifuge bile and dilute 1:10

**HPLC VARIABLES**
**Column:** 150 × 4.6 5 μm Ultrasphere ODS
**Mobile phase:** 24:76 MeCN:20 mM ammonium acetate adjusted to pH 5 with glacial acetic acid
**Flow rate:** 1
**Injection volume:** 20
**Detector:** UV 214

**CHROMATOGRAM**
**Retention time:** 4.8
**Limit of detection:** 500 ng/mL

**OTHER SUBSTANCES**
**Also analyzed:** ampicillin, azlocillin, aztreonam, cefmenoxime, cefoperazone, cefsulodin, cefotaxime, ceftazidime, ceftriaxone, cloxacillin, desacetylcefotaxime, mezlocillin, piperacillin, ticarcillin

**KEY WORDS**
serum

**REFERENCE**
Jehl,F.; Birckel,P.; Monteil,H. Hospital routine analysis of penicillins, third-generation cephalosporins and aztreonam by conventional and high-speed high-performance liquid chromatography, *J.Chromatogr.*, **1987**, *413*, 109–119.

**SAMPLE**
**Matrix:** blood

**Sample preparation:** Condition a Bond Elut phenyl SPE cartridge with 3 mL MeOH and 3 mL buffer. 1 mL Plasma + 2 mL buffer, vortex, add to SPE cartridge, wash with buffer, elute with 500 μL MeOH:10 mM pH 5.2 potassium phosphate buffer 90:10, inject a 25 μL aliquot. (Buffer was 121 g Trizma base in 1 L water, adjust pH to 7.0 with concentrated HCl. Dilute 1:100 to obtain the 10 mM buffer.)

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**HPLC VARIABLES**
**Column:** 150 × 3.9 4 μm Nova-Pak phenyl
**Mobile phase:** MeOH:10 mM pH 5.2 potassium phosphate buffer 20:80 (Buffer was 1 M $KH_2PO_4$ adjusted to pH 5.2 with 5 M KOH, dilute 1:100 with water to give working buffer.)
**Column temperature:** 50
**Flow rate:** 0.9
**Injection volume:** 25
**Detector:** UV 225

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**CHROMATOGRAM**
**Retention time:** 412
**Internal standard:** penicillin G

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**OTHER SUBSTANCES**
**Extracted:** cefteran (ceftetrane)

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**KEY WORDS**
plasma; SPE; method stated to be applicable to urine (no details); penicillin G is IS

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**REFERENCE**
Hicks,C.M.; Powell,M.L. Rapid analysis of ceftetrame in human plasma using sorbent extraction and high-performance liquid chromatography, *J.Chromatogr.*, **1989**, *497*, 349–354.

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**SAMPLE**
**Matrix:** blood
**Sample preparation:** Condition a 55 × 5 100-200 mesh AG 50W-X8 ($H^+$) column (Bio-Rad) with 10 mL MeCN:water 50:50. 600 μL Serum + 600 μL MeCN, vortex for 1 min, centrifuge at 2000 g for 5 min, add a 1 mL aliquot of the supernatant to the column, discard the first 200 μL effluent, collect the rest of the effluent. Remove a 450 μL aliquot and add it to 50 μL 10% sodium carbonate solution, heat at 60° for 1 h (to hydrolyse the β-lactam ring), cool in an ice bath. Remove a 100 μL aliquot and add it to 15 μL 200 mM pH 6.0 phosphate buffer, add 35 μL 80 mM 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole in MeCN, heat at 60° for 10 min, cool in an ice bath, add 30 μL 1 M HCl, inject a 5-10 μL aliquot.

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**HPLC VARIABLES**
**Column:** 150 × 4.6 ODS-80TM (Tosoh)
**Mobile phase:** MeOH:100 mM pH 3.0 phosphate buffer 40:60
**Flow rate:** 1
**Injection volume:** 5-10
**Detector:** F ex 470 em 530

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**CHROMATOGRAM**
**Retention time:** 10
**Limit of detection:** 30 ng/mL

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**OTHER SUBSTANCES**
**Extracted:** methicillin, piperacillin

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**KEY WORDS**
derivatization; serum; SPE

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**REFERENCE**
Iwaki,K.; Okumura,N.; Yamazaki,M.; Nimura,N.; Kinoshita,T. Precolumn derivatization technique for high-performance liquid chromatographic determination of penicillins with fluorescence detection, *J.Chromatogr.*, **1990**, *504*, 359–367.

## SAMPLE

**Matrix:** blood

**Sample preparation:** Condition a Baker C18 SPE cartridge with 5 mL water and 5 mL 2% NaCl, do not allow to run dry. 2 mL Plasma + 120 μL 5 μg/mL penicillin V + 30 mL water + 2 mL 170 mM sulfuric acid +2 mL 5% sodium tungstate solution, vortex for 30 s, centrifuge at 2200 g for 10 min, filter supernatant (GF/B glass fiber filter), add 10 mL 20% NaCL, mix, add to SPE cartridge at 3 mL/min, wash with 5 mL 2% NaCl, wash with 5 mL water, draw air through cartridge for 5 min, elute with 500 μL elution solution. Add 500 μL derivatization reagent to the eluate, vortex for 20 s, allow to react at 65° for 30 min, cool to room temperature, vortex, filter (0.45 μm), inject 50-100 μL aliquots. (Prepare derivatization reagent by dissolving 34.45 g 1,2,4-triazole in 150 mL water, add 25 mL 10 mM mercuric chloride solution, mix, adjust the pH to 9.0 ± 0.5 with 5 M NaOH, dilute to 250 mL with water. Prepare elution solution by mixing 60 mL MeCN and 5 mL buffer and making up to 100 mL with water. The buffer was 0.994 g $Na_2HPO_4$ + 1.794 g $NaH_2PO_4.H_2O$ in 100 mL water, pH 6.5.)

## HPLC VARIABLES

**Column:** 150 × 3.9 4 μm Nova-Pak C18

**Mobile phase:** MeCN:buffer 25:75 (Buffer contained 4.969 g $Na_2HPO_4$ + 8.969 g $NaH_2PO_4.H_2O$ + 2.482 g anhydrous sodium thiosulfate per liter.)

**Flow rate:** 1

**Injection volume:** 50-100

**Detector:** UV 325

## CHROMATOGRAM

**Retention time:** 4.5

**Internal standard:** penicillin V (5.8)

**Limit of detection:** 5 ng/mL

## KEY WORDS

plasma; cow; SPE; derivatization

## REFERENCE

Boison,J.O.; Korsrud,G.O.; MacNeil,J.D.; Keng,L.; Papich,M. Determination of penicillin G in bovine plasma by high-performance liquid chromatography after pre-column derivatization, *J.Chromatogr.*, **1992**, *576*, 315–320.

## SAMPLE

**Matrix:** blood, CSF

**Sample preparation:** 200 μL Serum, plasma, or CSF + 300 μL reagent. Flush column A to waste with 500 μL 500 mM ammonium sulfate, inject sample onto column A, flush column A to waste with 500 μL 500 mM ammonium sulfate, elute the contents of column A onto column B with mobile phase, monitor the effluent from column B. (Reagent was 8.05 M guanidine hydrochloride and 1.02 M ammonium sulfate in water.)

## HPLC VARIABLES

**Column:** A 30 × 2.1 40 μm preparative grade C18 (Analytichem); B 250 × 4.6 10 μm Partisil C8

**Mobile phase:** Gradient. A was 50 mM pH 4.5 $KH_2PO_4$. B was MeCN:isopropanol 80:20. A:B 90:10 for 1 min, to 30:70 over 15 min, maintain at 30:70 for 4 min.

**Column temperature:** 50

**Flow rate:** 1.5

**Detector:** UV 280 for 5 min then UV 254

## CHROMATOGRAM

**Retention time:** 10.28

**Internal standard:** heptanophenone (19.2)

## OTHER SUBSTANCES

**Extracted:** acetazolamide, ampicillin, bromazepam, caffeine, carbamazepine, chloramphenicol, chlorothiazide, diazepam, droperidol, ethionamide, furosemide, isoniazid, methadone, phenobarbital, phenytoin, prazepam, propoxyphene, pyrazinamide, rifampin, trimeprazine, trimethoprim

**KEY WORDS**
plasma; serum; column-switching

**REFERENCE**
Seifart,H.I.; Kruger,P.B.; Parkin,D.P.; van Jaarsveld,P.P.; Donald,P.R. Therapeutic monitoring of antituberculosis drugs by direct in-line extraction on a high-performance liquid chromatography system, *J.Chromatogr.*, **1993**, *619*, 285–290.

**SAMPLE**
**Matrix:** blood, CSF, gastric contents, urine
**Sample preparation:** 200 μL Serum, urine, CSF, or gastric fluid + 300 μL reagent. Flush column A to waste with 500 μL 500 mM ammonium sulfate, inject sample onto column A, flush column A to waste with 500 μL 500 mM ammonium sulfate, backflush the contents of column A onto column B with mobile phase, monitor the effluent from column B. (Reagent was 8.05 M guanidine HCl and 1.02 M ammonium sulfate in water.)

**HPLC VARIABLES**
**Column:** A 40 μm preparative grade C18 (Analytichem); B 75 × 2.1 pellicular C18 (Whatman) + 250 × 4.6 5 μm C8 end-capped (Whatman)
**Mobile phase:** Gradient. A was 50 mM pH 4.5 $KH_2PO_4$. B was MeCN:isopropanol 80:20. A:B 90:10 for 1 min, to 30:70 over 20 min.
**Column temperature:** 50
**Flow rate:** 1.5
**Detector:** UV 220

**CHROMATOGRAM**
**Retention time:** 8.5
**Internal standard:** heptanophenone (19)

**OTHER SUBSTANCES**
**Extracted:** acetaminophen, allobarbital, azinphos, barbital, brallobarbitone, bromazepam, butethal, caffeine, carbamazepine, carbaryl, cephaloridine, chloramphenicol, chlordiazepoxide, chlorothiazide, chlorvinphos, clothiapine, cocaine, coomassie blue, desipramine, diazepam, diphenhydramine, dipipanone, ethylbromphos, flufenamic acid, formothion, griseofulvin, indomethacin, lidocaine, lorazepam, malathion, medazepam, midazolam, oxazepam, paraoxon, pentobarbital, prazepam, propoxyphene, prothiophos, quinine, salicylic acid, secobarbital, strychnine, sulfamethoxazole, theophylline, thiopental, thioridazine, trimethoprim

**KEY WORDS**
serum; column-switching

**REFERENCE**
Kruger,P.B.; Albrecht,C.F.De V.; Jaarsveld,P.P. Use of guanidine hydrochloride and ammonium sulfate in comprehensive in-line sorption enrichment of xenobiotics in biological fluids by high-performance liquid chromatography, *J.Chromatogr.*, **1993**, *612*, 191–198.

**SAMPLE**
**Matrix:** blood, CSF, urine
**Sample preparation:** Plasma. Add 500 μL MeCN to 500 μL plasma while mixing on a Whirlmixer, rotate for 10 min, centrifuge at 1000 g for 5 min. Remove a 700 μL aliquot of the supernatant and ad it to 3.5 mL dichloromethane, mix for 30 s, centrifuge at 1000 g for 1 min, inject a 20 μL aliquot of the aqueous layer. Urine. Dilute with Sörensen buffer, inject an aliquot. CSF. Inject an aliquot directly.

**HPLC VARIABLES**
**Column:** 100 × 3 5 μm MOS-Hypersil C8
**Mobile phase:** MeCN:MeOH:buffer 12:26:62 containing 3 mM tetrabutylammonium bromide (Buffer was 5 mM pH 5.0 sodium acetate.)
**Column temperature:** 22
**Flow rate:** 1
**Injection volume:** 20

**Detector:** UV 231

**CHROMATOGRAM**
**Retention time:** 3
**Limit of detection:** 500 ng/mL

**OTHER SUBSTANCES**
**Extracted:** probenecid

**KEY WORDS**
plasma

**REFERENCE**
van Gulpen,C.; Brokerhof,A.W.; van der Kaay,M.; Tjaden,U.R.; Mattie,H. Determination of benzylpenicillin and probenecid in human body fluids by high-performance liquid chromatography, *J.Chromatogr.*, **1986**, *381*, 365–372.

**SAMPLE**
**Matrix:** blood, tissue, urine
**Sample preparation:** Plasma. 50 μL Plasma + 50 μL IS solution + 50 μL MeCN, mix for 30 s, centrifuge at 5000 g for 15 min. Inject an aliquot of the supernatant. Urine. Mix 100 μL IS solution with 200 μL MeCN and 100 μL urine for 30 s, centrifuge at 5000 g for 15 min. Inject an aliquot. Tissue. Weight out finely chopped tissue and suspend it in 200 μL water. Add 100 μL 100 μg/mL IS, sonicate for 60 s. Add 200 μL MeCN, vortex for 30 s, centrifuge at 10000 g for 15 min. Inject an aliquot.

**HPLC VARIABLES**
**Guard column:** 15 × 3.2 7 μm Newguard C18 (Alltech)
**Column:** 250 × 4.6 5 μm Alltima C18 (Alltech)
**Mobile phase:** MeCN:50 mM pH 5.0 sodium dihydrogen phosphate 30:70
**Flow rate:** 1.0
**Detector:** UV 214

**CHROMATOGRAM**
**Retention time:** 6.4
**Internal standard:** dicloxacillin (13.9)
**Limit of quantitation:** 800 ng/mL (plasma), 1 μg/mL (urine), 5 μg/g (tissue)

**OTHER SUBSTANCES**
**Extracted:** flucloxacillin

**KEY WORDS**
plasma; muscle; rat; pharmacokinetics

**REFERENCE**
Cross,S.E.; Thompson,M.J.; Roberts,M.S. Distribution of systemically administered ampicillin, benzylpenicillin, and flucloxacillin in excisional wounds in diabetic and normal rats and effects of local topical vasodilator treatment, *Antimicrob.Agents Chemother.*, **1996**, *40*, 1703–1710.

**SAMPLE**
**Matrix:** blood, urine
**Sample preparation:** Plasma, serum. 200 μL Plasma or serum + 200 μL 50 mM pH 6.0 sodium phosphate buffer + 800 μL MeCN, vortex for 30 s, centrifuge at 3000 g for 10 min. Remove the clear supernatant and add it to 2 mL dichloromethane, mix for 30 s, centrifuge at 3000 g for 10 min, inject a 25 μL aliquot of the upper aqueous layer. Urine. 100 μL Urine + 9.9 mL 50 mM pH 6.0 sodium phosphate buffer, inject a 25 μL aliquot.

**HPLC VARIABLES**
**Guard column:** 15 × 3.2 7 μm Brownlee C 18 guard column
**Column:** 250 × 4.6 5 μm Hypersil ODS (Keystone)

**Mobile phase:** Gradient. A was MeCN:10 mM sodium phosphate adjusted to pH 2.7 with concentrated phosphoric acid 3:97. B was MeCN:10 mM sodium phosphate adjusted to pH 2.7 with concentrated phosphoric acid 90:10. A:B from 95:5 to 50:50 over 9 min and then to 95:5 over 1 min.
**Flow rate:** 1.5
**Injection volume:** 25
**Detector:** UV 220

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**CHROMATOGRAM**
**Retention time:** 12.5
**Internal standard:** penicillin G

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**OTHER SUBSTANCES**
**Extracted:** piperacillin, tazobactam
**Simultaneous:** amoxicillin, ampicillin, cefoperazine, cefometazole, cefotaxime, cefotetan, cefuroxime, mezlocillin

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**KEY WORDS**
plasma; serum; penicillin G is IS

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**REFERENCE**
Ocampo,A.P.; Hoyt,K.D.; Wadgaonkar,N.; Carver,A.H.; Puglisi,C.V. Determination of tazobactam and piperacillin in human plasma, serum, bile and urine by gradient elution reversed-phase high-performance liquid chromatography, *J.Chromatogr.*, **1989**, *496*, 167–179.

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**SAMPLE**
**Matrix:** cell suspensions
**Sample preparation:** 300 μL Cell suspension + 300 μL MeCN, vortex, centrifuge, inject a 10 μL aliquot.

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**HPLC VARIABLES**
**Column:** 80 × 4 Nucleosil 120 3 C18
**Mobile phase:** MeCN:20 mM phosphoric acid 25:75
**Injection volume:** 10
**Detector:** UV 210

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**CHROMATOGRAM**
**Retention time:** 3.1

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**REFERENCE**
Kersten,A.; Poitschek,C.; Rauch,S.; Aberer,E. Effects of penicillin, ceftriaxone, and doxycycline on morphology of *Borrelia burgdorferi*, *Antimicrob.Agents Chemother.*, **1995**, *39*, 1127–1133.

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**SAMPLE**
**Matrix:** cheese, milk, yogurt
**Sample preparation:** Condition a 6 mL 500 mg Bond Elut C18 SPE cartridge with 20 mL MeOH, 20 mL water, and 10 mL 2% NaCl, do not allow to go dry. 5 mL Milk (or 5 g yogurt or cottage cheese + 4 mL 1 M pH 6 phosphate buffer) + 20 μL 20 μg/mL penicillin V in water + 25 mL water + 4 mL 170 mM sulfuric acid + 40 mL 5% sodium tungstate, vortex for 30 s, centrifuge at 1500 g for 10 min, remove the supernatant, add 10 mL 20% NaCl to the residue, vortex for 10 s, centrifuge. Combine the supernatants and add them to the SPE cartridge, wash with 10 mL 2% NaCl, wash with 10 mL water, elute with 750 μL MeCN:200 mM ammonium acetate:water 60:5:35, filter (Acro 0.45 μm), inject a 50-100 μL aliquot of the filtrate.

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**HPLC VARIABLES**
**Column:** 150 × 3.9 4 μm Nova-Pak C18
**Mobile phase:** Gradient. A was MeCN. B was MeCN:150 mM ammonium acetate 10:90. A:B 0:100 for 10 min, to 30:70 over 10 min, return to initial conditions over 10 min.
**Flow rate:** 0.9
**Injection volume:** 50-100

**Detector:** MS, VG Trio II, probe tip 255°, source 180°, thermospray/plasmaspray, m/z 335, m/z 160

**CHROMATOGRAM**
**Internal standard:** penicillin V
**Limit of detection:** 5 ng/mL

**KEY WORDS**
cow; SPE

**REFERENCE**
Boison,J.O.K.; Keng,L.J.-Y.; MacNeil,J.D. Analysis of penicillin G in milk by liquid chromatography, *J.AOAC Int.*, **1994**, *77*, 565–570.

**SAMPLE**
**Matrix:** fermentation solutions
**Sample preparation:** Adjust pH of fermentation broth to 7, centrifuge at 8000 g for 10 min, add MeCN, centrifuge, add dichloromethane to the supernatant, vortex for 10 s, shake for 15 min, centrifuge at 8000 g for 15 min. Add 1 mL of the aqueous layer to 100 μL reagent, heat at 50° for 50 min, cool in an ice bath, inject a 20 μL aliquot. (Prepare reagent by dissolving 4.125 g imidazole in 2.5 mL water, add 1 mL HCl, add 500 μL 110 mM mercury(II) chloride, add 1.5 mL HCl. Recrystallize imidazole twice from isopropanol.)

**HPLC VARIABLES**
**Guard column:** 10 × 4 5 μm Spherisorb C18
**Column:** 20 × 4.6 5 μm Spherisorb C18 S5ODS2
**Mobile phase:** Gradient. MeCN:buffer from 16.5:83.5 to 31.5:68.5 over 17 min (Buffer was 10 mM $NaH_2PO_4$ containing 10 mM EDTA, adjusted to pH 6.5 with 2 M NaOH.)
**Flow rate:** 2
**Injection volume:** 20
**Detector:** UV 325

**CHROMATOGRAM**
**Retention time:** 13
**Limit of detection:** 1 μg/mL

**OTHER SUBSTANCES**
**Extracted:** methicillin, penicillin V, penicillin X

**KEY WORDS**
derivatization

**REFERENCE**
Rogers,M.E.; Adlard,M.W.; Saunders,G.; Holt,G. High-performance liquid chromatographic determination of penicillins following derivatization to mercury-stabilized penicillenic acids, *J.Liq.Chromatogr.*, **1983**, *6*, 2019–2031.

**SAMPLE**
**Matrix:** fermentation solutions
**Sample preparation:** Filter (0.22 μm) fermentation broth. Mix 980 μL filtrate with 20 μL 200 mM benzoic anhydride in MeCN at room temperature for 3 min, add 100 μL reagent, mix, heat at 45° for 1 h, cool to room temperature, inject a 40 μL aliquot. (Prepare reagent by dissolving 6.76 g 1-hydroxybenzotriazole hydrate in 10 mL water, add 2.5 mL mercury(II) chloride solution (no concentration given), adjust pH to 9.2 with 4 M NaOH, make up to 25 mL.)

**HPLC VARIABLES**
**Guard column:** 10 × 4 5 μm Spherisorb S5ODS2
**Column:** 259 × 4.9 5 μm Spherisorb S5ODS2
**Mobile phase:** Gradient. MeCN:20 mM pH 6.5 potassium phosphate buffer:20 mM sodium thiosulfate from 10:45:45 to 25:37.5:37.5 over 25 min, maintain at 25:37.5:37.5 for 10 min (The mobile phase flowed through a 50 × 4 column of 5 μm Spherisorb S5ODS2 before the injector.)

**Flow rate:** 1 for 25 min then 1.2
**Injection volume:** 40
**Detector:** UV 328

**CHROMATOGRAM**
**Retention time:** 24
**Limit of detection:** 1 ng/mL

**OTHER SUBSTANCES**
**Extracted:** 6-aminopenicillanic acid, ampicillin, penicillin K, penicillin V, penicillin X

**KEY WORDS**
derivatization

**REFERENCE**
Shah,A.J.; Adlard,M.W.; Holt,G. Determination of natural penicillins in fermentation media by high-performance liquid chromatography using precolumn derivatization with 1-hydroxybenzotriazole, *Analyst*, **1988**, *113*, 1197–1200.

**SAMPLE**
**Matrix:** fermentation solutions
**Sample preparation:** Centrifuge fermentation broth at 4° at 5000 g for 20 min, filter (0.45 μm) a 1 mL aliquot of the supernatant, add 50 μL 1 M NaOH to the filtrate. Remove a 50 μL aliquot and add it to 50 μL 3 mM N-dansylaziridine (Sigma) in dioxane (Caution! Dioxane is a carcinogen!), heat at 100° for 30 min, cool to room temperature, inject a 20 μL aliquot.

**HPLC VARIABLES**
**Guard column:** 50 × 4.6 5 μm Spherisorb C18
**Column:** 250 × 4.6 5 μm Spherisorb C18
**Mobile phase:** Gradient. MeCN:20 mM pH 4.4 acetate buffer containing 0.5 mM EDTA from 19:81 to 23:77 over 15 min, to 40:60 over 10 min, to 65:35 over 2.5 min.
**Flow rate:** 1
**Injection volume:** 20
**Detector:** F ex 339 em 540

**CHROMATOGRAM**
**Retention time:** 24
**Limit of detection:** 50 ng/mL

**OTHER SUBSTANCES**
**Extracted:** δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine, 6-aminopenicillanc acid, isopenicillin N

**KEY WORDS**
derivatization

**REFERENCE**
Orford,C.D.; Perry,D.; Adlard,M.W. The determination of naturally produced penicillins and their biosynthetic precursors using pre-column derivatisation with dansylaziridine, *J.Liq.Chromatogr.*, **1991**, *14*, 2665–2684.

**SAMPLE**
**Matrix:** formulations
**Sample preparation:** Blend tablets and capsules with water in a high-speed blender for 5 min, filter, dilute with mobile phase, inject a 20 μL aliquot. Dilute oral suspensions and injections with mobile phase, inject a 20 μL aliquot.

**HPLC VARIABLES**
**Guard column:** 70 mm long Co:Pell ODS
**Column:** 300 × 4.6 10 μm Chromegabond C18 (E.S. Industries)
**Mobile phase:** MeCN:MeOH:10 mM $KH_2PO_4$ 19:11:70
**Flow rate:** 1

**Injection volume:** 20
**Detector:** UV 225

**CHROMATOGRAM**
**Retention time:** 6.2
**Limit of detection:** 1700 ng/mL

**OTHER SUBSTANCES**
**Simultaneous:** amoxicillin, ampicillin, cloxacillin, dicloxacillin, methicillin, nafcillin, oxacillin, penicillin V

**KEY WORDS**
tablets; capsules; oral suspensions; injections

**REFERENCE**
Briguglio,G.T.; Lau-Cam,C.A. Separation and identification of nine penicillins by reverse phase liquid chromatography, *J.Assoc.Off.Anal.Chem.*, **1984**, *67*, 228–231.

**SAMPLE**
**Matrix:** formulations
**Sample preparation:** Dilute with water, inject an aliquot.

**HPLC VARIABLES**
**Column:** 150 × 3.9 5 μm Nova Pak C18
**Mobile phase:** MeOH:buffer 50:50 (Buffer was 5 mM pH 7.5 containing 1.3 mM tetrabutylammonium hydroxide.)
**Flow rate:** 0.5
**Detector:** UV 254

**CHROMATOGRAM**
**Retention time:** 2.4

**OTHER SUBSTANCES**
**Simultaneous:** degradation products

**KEY WORDS**
injections; water; stability-indicating

**REFERENCE**
Stiles,M.L.; Tu,Y.H.; Allen,L.V.,Jr. Stability of cefazolin sodium, cefoxitin sodium, ceftazidime, and penicillin G sodium in portable pump reservoirs, *Am.J.Hosp.Pharm.*, **1989**, *46*, 1408–1412.

**SAMPLE**
**Matrix:** milk
**Sample preparation:** 50 g Milk + 2 drops penicillinase (Difco Laboratories), let stand 1 h at 37°, add 50 mL MeCN, shake vigorously for 1 min, centrifuge at 9000 g for 10 min, decant, add 5 g NaCl, swirl to dissolve, add 100 mL dichloromethane, shake for 1 min, centrifuge at 1000 g for 10 min. Remove top aqueous layer and extract organic layer with 25 mL 10% NaCl by shaking and centrifuging as before. Combine aqueous layers, add 1 mL 0.3% mercuric chloride in water, let stand 30 min, add 1 mL 2 M HCl, extract with three 50 mL portions of dichloromethane by shaking each portion for 1 min and centrifuging at 1000 g for 10 min, filter dichloromethane extracts through 30 g anhydrous sodium sulfate, evaporate to dryness under reduced pressure at 35°, if water remains add 5-10 mL MeOH to flask and complete evaporation. Dissolve residue in 1 mL 10% acetic acid, add 0.5 mL 0.08% dansyl hydrazine in 10% acetic acid, let stand 90 min to overnight in the dark, transfer reaction mixture to a separatory funnel with three 25 mL portions of dichloromethane, add 5 mL 2 M HCl, shake for 1 min, wash organic layer with 5 mL 5% $NaHCO_3$ solution, filter through 10-20 g anhydrous sodium sulfate. Extract acid aqueous layer again with 25 mL dichloromethane. Combine dichloromethane layers and evaporate to dryness at 35° under reduced pressure. Dissolve residue in 2 mL IS solution, inject a 20 μL aliquot. (Prepare IS solution by dissolving 10 μL benzaldehyde in 100 mL dichloromethane, evaporate 1 mL to dryness under reduced pressure, dissolve res-

idue in 1 mL 10% acetic acid, add 0.5 mL 0.08% dansyl hydrazine in 10% acetic acid, let stand 90 min to overnight in the dark, transfer reaction mixture to a separatory funnel with three 25 mL portions of dichloromethane, add 5 mL 2 M HCl, shake for 1 min, wash organic layer with 5 mL 5% $NaHCO_3$ solution, filter through 10-20 g anhydrous sodium sulfate. Extract acid aqueous layer again with 25 mL dichloromethane. Combine dichloromethane layers and evaporate to dryness at 35° under reduced pressure. Dissolve residue in 100 mL MeCN then dilute an aliquot 1:4 with MeCN.)

**HPLC VARIABLES**
**Column:** 250 × 4 10 μm Lichrosorb RP-18
**Mobile phase:** MeCN:water 58:42
**Flow rate:** 1
**Injection volume:** 20
**Detector:** F ex 254 em 500 filter

**CHROMATOGRAM**
**Retention time:** 5.74
**Internal standard:** benzaldehyde (derivatized) (12.18)
**Limit of detection:** 5 ng/g

**OTHER SUBSTANCES**
**Extracted:** penicillin V, phenethicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin
**Interfering:** methicillin

**KEY WORDS**
derivatization

**REFERENCE**
Munns,R.K.; Shimoda,W.; Roybal,J.E.; Vieira,C. Multiresidue method for determination of eight neutral β-lactam penicillins in milk by fluorescence-liquid chromatography, *J.Assoc.Off.Anal.Chem.*, **1985**, *68*, 968–971.

**SAMPLE**
**Matrix:** milk
**Sample preparation:** Condition a Sep-Pak C18 SPE cartridge with 20 mL MeOH, 20 mL water, and 2 mL 2% NaCl. Pass through 30 g filtered (glass-wool plug) milk at 2 mL/min, wash with 5 mL water, wash with 10 mL MeOH:water:20% NaCl 10:80:10 containing 20 mM 18-crown-6, elute with 10 mL 15% (v/v) MeOH, inject a 100 μL aliquot.

**HPLC VARIABLES**
**Guard column:** 50 × 2.1 Permaphase ETH (Du Pont)
**Column:** 150 × 4.3 LiChrosorb RP-18
**Mobile phase:** MeOH:water:0.2 M pH 4.0 phosphate buffer 25:65:10 containing 11 mM sodium 1-heptanesulfonate
**Column temperature:** 45
**Flow rate:** 1
**Injection volume:** 100
**Detector:** UV 210

**CHROMATOGRAM**
**Retention time:** 9
**Limit of detection:** 30 ng/g

**OTHER SUBSTANCES**
**Extracted:** ampicillin, penicillin V

**KEY WORDS**
cow; SPE

### REFERENCE

Terada,H.; Sakabe,Y. Studies on residual antibacterials in foods. IV. Simultaneous determination of penicillin G, penicillin V and ampicillin in milk by high-performance liquid chromatography, *J.Chromatogr.*, **1985**, *348*, 379–387.


### SAMPLE

**Matrix:** milk

**Sample preparation:** 500 μL Milk + 500 μL MeCN:MeOH:water 40:20:40, vortex for 10-15 s, filter (Amicon Centricon-10, 10000 dalton cut-off) while centrifuging at 2677 g for 30 min, inject a 10-60 μL aliquot of the ultrafiltrate.

### HPLC VARIABLES

**Column:** 220 × 2.1 5 μm Spheri-5 phenyl

**Mobile phase:** MeCN:buffer 25:75 (Buffer was 2.5 mM octanesulfonate, 2.5 mM dodecanesulfonate, 0.5% 85% phosphoric acid, and 0.5% triethylamine.)

**Column temperature:** 40

**Flow rate:** 0.3-0.5

**Injection volume:** 10-60

**Detector:** UV 210

### CHROMATOGRAM

**Retention time:** 6.3

**Limit of detection:** 10 ppb

### KEY WORDS

cow; ultrafiltrate


### REFERENCE

Tyczkowska,K.; Voyksner,R.D.; Aronson,A.L. Development of an analytical method for penicillin G in bovine milk by liquid chromatography with ultraviolet-visible detection and confirmation by mass spectrometric detection, *J.Chromatogr.*, **1989**, *490*, 101–113.


### SAMPLE

**Matrix:** milk

**Sample preparation:** Condition a 6 mL 500 mg Bond Elut C18 SPE cartridge with 20 mL MeOH, 20 mL water, and 10 mL 2% NaCl, do not allow to go dry. 5 mL Milk (or 5 g yogurt or cottage cheese + 4 mL 1 M pH 6 phosphate buffer) + 20 μL 20 μg/mL penicillin V in water + 25 mL water + 4 mL 170 mM sulfuric acid + 40 mL 5% sodium tungstate, vortex for 30 s, centrifuge at 1500 g for 10 min, remove the supernatant, add 10 mL 20% NaCl to the residue, vortex for 10 s, centrifuge. Combine the supernatants and add them to the SPE cartridge, wash with 10 mL 2% NaCl, wash with 10 mL water, elute with 1 mL MeCN:200 mM pH 6.5 sodium phosphate buffer:water 60:5:35. Add 1 mL reagent to the eluate, vortex for 10 s, heat at 65° for 30 min, cool to room temperature, vortex, filter (Acro 0.45 μm), inject a 50-100 μL aliquot of the filtrate. (Prepare reagent by dissolving 34.45 g 1,2,4-triazole in 150 mL water, add 25 mL 10 mM mercuric chloride solution, mix, adjust pH to 9.0 ± 0.5 with 5 M NaOH, make up to 250 mL with water.)

### HPLC VARIABLES

**Column:** 150 × 3.9 4 μm Nova-Pak C18

**Mobile phase:** MeCN:buffer 25:75 (Buffer was 4.696 g $Na_2HPO_4$, 8.969 g $NaH_2PO_4.H_2O$, and 2.482 g anhydrous sodium thiosulfate in 1 L water.)

**Flow rate:** 0.8

**Injection volume:** 50-100

**Detector:** UV 325

### CHROMATOGRAM

**Retention time:** 5.5

**Internal standard:** penicillin V (7)

**Limit of detection:** 3 ng/mL

### KEY WORDS

derivatization; cow; SPE

### REFERENCE

Boison,J.O.K.; Keng,L.J.-Y.; MacNeil,J.D. Analysis of penicillin G in milk by liquid chromatography, *J.AOAC Int.*, **1994**, *77*, 565–570.

### SAMPLE

**Matrix:** milk

**Sample preparation:** Add 2 volumes MeCN to milk, stand 5 min, decant aqueous portion, suction filter, extract with an equal volume of 1:1 methylene chloride:hexane, centrifuge aqueous phase at 3000 rpm for 10 min. Dilute 3:1 with 20 mM sodium acetate buffer and filter (0.2 μm nylon). Inject 50 μL onto column with mobile phase A, run mobile phase A for 30 min and elute to waste. After 30 min switch to mobile phase B and elute through detector.

### HPLC VARIABLES

**Column:** 100 × 8 Radial-Pak 10 μm μBondapak C18

**Mobile phase:** A 20 mM sodium acetate buffer; B Gradient. MeCN:MeOH:20 mM sodium acetate buffer from 15:10:75 to 30:0:70 over 15 min and hold at 30:0:70

**Flow rate:** A 3; B 2

**Injection volume:** 50

**Detector:** E, Waters 464 pulsed electrochemical detector using a thin layer cell with a Ag/AgCl reference electrode. E1 = 1300 mV for 0.166 s, E2 = 1500 mV for 0.166 s, E3 = -200 mV for 0.333 s.

### CHROMATOGRAM

**Retention time:** 9

**Limit of detection:** 0.2 ppm

### OTHER SUBSTANCES

**Simultaneous:** penicillin V, ampicillin, methicillin, oxacillin, cloxacillin, nafcillin, dicloxacillin.

### REFERENCE

Kirchmann,E.; Earley,R.L.; Welch,L.E. The electrochemical detection of penicillins in milk, *J.Liq.Chromatogr.*, **1994**, *17*, 1755–1772.

### SAMPLE

**Matrix:** milk

**Sample preparation:** Condition a 1 mL 100 mg Bond Elut C2 SPE cartridge with 1 mL MeCN and two 1 mL portions of water, suck dry for 5 s. 1 mL Milk + 200 μL water + 10 mL acetone, mix for 10 s, centrifuge at 3000 rpm for 3 min. Remove the organic layer and evaporate it to 600 μL under a stream of nitrogen at 45°, add 1 mL hexane, shake vigorously for 5 s, centrifuge for 2 min, discard the hexane layer, repeat the hexane wash, evaporate the aqueous layer to dryness, reconstitute the residue in 350 μL MeCN:water 20:80, add slowly to the SPE cartridge, suck dry for 5 s, wash with two 50 μL portions of MeCN:water 10:90, suck dry for 5 s, elute with four 100 μL portions of MeCN:water 20:80. Centrifuge the eluate at 3000 rpm for 3 min, inject a 125 μL aliquot.

### HPLC VARIABLES

**Guard column:** 20 × 4.6 5 μm Supelcosil LC-C18 DB

**Column:** 250 × 4.6 5 μm Supelcosil LC-C18 DB

**Mobile phase:** MeCN:buffer 34:66 (Prepare buffer by dissolving 1.78 g $Na_2HPO_4.2H_2O$ and 4.45 g sodium 1-heptanesulfonate in 750 mL water, adjust pH to 2.15 with 5 M phosphoric acid, make up to 1 L with water.)

**Column temperature:** 35

**Flow rate:** 0.9 for 3 min, 0.6 for 10 min, 2 for 2 min

**Injection volume:** 125

**Detector:** UV 200

### CHROMATOGRAM

**Retention time:** 12

**Limit of detection:** 2 ng/mL

**Limit of quantitation:** 4 ng/mL

**KEY WORDS**
cow; SPE

**REFERENCE**
Hormazal,V.; Yndestad,M. Detection of benzylpenicillin in milk by HPLC, *J.Liq.Chromatogr.*, **1995**, *18*, 2469–2474.

**SAMPLE**
**Matrix:** milk
**Sample preparation:** 10 mL Milk + 2 mL 200 mM tetraethylammonium chloride, stir, slowly add 38 mL MeCN over 30 s, let stand for 5 min, decant the supernatant through a plug of glass wool. 40 mL Filtrate + 1 mL water, evaporate under reduced pressure to 1-2 mL, make up to 4 mL with water, filter (0.45 μm polyvinylidene difluoride). Inject 2 mL into an LC system (150 × 4.6 5 μm Supelcosil LC-18; MeCN:10 mM $KH_2PO_4$ 0:100 for 3 min, to 40:60 over 27 min, to 0:100 over 1 min; 1 mL/min; UV 210 and 295), collect a 1.5 mL fraction at retention time for penicillin G (23 min), evaporate to 1 mL, inject a 200 μL aliquot.

**HPLC VARIABLES**
**Column:** 150 × 4.6 5 μm Supelcosil LC-18-DB
**Mobile phase:** MeCN:buffer 29:71 (Buffer was 3.3 mM phosphoric acid and 6.7 mM $KH_2PO_4$.)
**Flow rate:** 1
**Injection volume:** 200
**Detector:** UV 210

**CHROMATOGRAM**
**Limit of quantitation:** 2-5 ppb

**OTHER SUBSTANCES**
**Also analyzed:** ampicillin, amoxicillin, cephapirin, ceftiofur, penicillin V, cloxacillin

**KEY WORDS**
cow

**REFERENCE**
Moats,W.A.; Harik-Khan,R. Liquid chromatographic determination of β-lactam antibiotics in milk: A multiresidue approach, *J.AOAC Int.*, **1995**, *78*, 49–54.

**SAMPLE**
**Matrix:** milk
**Sample preparation:** Condition a Bond Elut C8 SPE cartridge with 5 mL MeOH and 5 mL water. 20 mL Milk + 20 mL buffer, heat at 60° for 20 min or until milk curdles, centrifuge for 10 min, add the supernatant to the SPE cartridge, wash with two 2.5 mL portions of water, elute with 2.5 mL MeOH. Evaporate the eluate to dryness under a stream of nitrogen, extract the residue with three 100 μL portions of 50 mM pH 6.0 potassium phosphate buffer, filter (0.2 μm), inject an aliquot of the filtrate. (Buffer was 545 mL 100 mM citric acid, 455 mL 200 mM $Na_2HPO_4$, and 74.4 g EDTA, adjust to pH 4.5 with ammonium hydroxide, make up to 2 L with water.)

**HPLC VARIABLES**
**Column:** 250 × 4.6 10 μm Lichrosorb RP-8
**Mobile phase:** MeOH:50 mM pH 6.0 potassium phosphate buffer 35:65
**Flow rate:** 1
**Injection volume:** 200
**Detector:** UV 210 or Charm II assay

**CHROMATOGRAM**
**Retention time:** 15.33

**OTHER SUBSTANCES**
**Extracted:** ampicillin, ceftiofur, cephapirin, cloxacillin, dicloxacillin, nafcillin, oxacillin
**Simultaneous:** amoxicillin

**KEY WORDS**
SPE

**REFERENCE**
Zomer,E.; Quintana,J.; Saul,S.; Charm,S.E. LC-Receptograms: A method for identification and quantitation of β-lactams in milk by liquid chromatography with microbial receptor assay, *J.AOAC Int.*, **1995**, *78*, 1165–1172.

**SAMPLE**
**Matrix:** milk
**Sample preparation:** Condition a 500 mg tC18 SPE cartridge (Waters) with 20 mL MeOH, 20 mL water, and 10 mL 2% NaCl. Centrifuge 30 mL milk at 1500 g for 10 min. Dilute a 10 mL portion of the defatted milk with 20 mL water, add 200 μL 2 μg/mL penicillin V in pH 9.0 buffer, add 6 mL 170 mM sulfuric acid, add 5.6 mL 5% sodium tungstate, shake vigorously for 1 min, allow to stand for 5 min, check that the pH is in the range 4.6-4.8 (if it is outside this range start again using a different volume of sodium tungstate solution), centrifuge at 1500 g for 10 min, adjust the pH of the supernatant to 8.1-8.2 with 5 M and 0.1 M NaOH, filter (glass fiber) the clear liquid phase. Pass the filtrate through the SPE cartridge at 2 mL/min, wash with 2 mL water, dry by pulling air through the cartridge for 1 min, elute with 2 mL MeCN. Add 150 μL pH 9.0 buffer to the eluate and evaporate to about 100 μL under a stream of nitrogen at 45-50°, add 400 μL pH 9.0 buffer, add 75 μL reagent I, vortex for 30 s, let stand at room temperature for 10 min, use 500 μL water to transfer the mixture to a separatory funnel, add 20 mL dichloromethane, add 5 mL pH 2.45 buffer, shake for 1 min, let stand for no more than 5 min. Remove the organic layer and evaporate it to dryness under reduced pressure at 35-40°, dissolve the residue in 500 μL pH 9.0 buffer, add 75 μL reagent I, vortex for 30 s, let stand at room temperature for 10 min, add 450 μL reagent II, vortex for 1 min, heat at 55 ± 1° for 30 min, cool, filter (0.45 μm), inject a 150 μL aliquot. (Prepare pH 9.0 buffer by dissolving 0.34 g $KH_2PO_4$ in water, adjusting the pH to 9.0 with NaOH, and making up to 100 mL with water. Prepare pH 2.45 buffer by dissolving 2.72 g $KH_2PO_4$ in water, adjusting the pH to 2.45 with phosphoric acid, and making up to 100 mL with water. Prepare reagent 1 by dissolving 1.13 g benzoic anhydride in MeCN, make up to 25 mL with MeCN. Prepare reagent II by dissolving 6.905 g 1,2,4-triazole in 30 mL water and adding 5 mL 26 mM mercuric chloride in water, adjust pH to 9.0 ± 0.05 with 5 M NaOH, make up to 50 mL. Prepare reagents I and II 1-4 h before use. Silanize glassware with dichlorodimethylsilane.)

**HPLC VARIABLES**
**Column:** 150 × 3.9 4 μm Nova-Pak C18
**Mobile phase:** Gradient. A as MeCN:buffer 10:90. B was MeCN:buffer 30:70. A:B from 100:0 to 0:100 over 30 min, maintain at 0:100 for 13 min, return to initial conditions over 2 min, re-equilibrate at initial conditions for 5 min. (Prepare buffer by dissolving 9.938 g $Na_2HPO_4$, 17.938 g $NaH_2PO_4.H_2O$, and 4.964 g sodium thiosulfate in water, make up to 2 L with water, pH 6.5.)
**Column temperature:** 30
**Flow rate:** 1
**Injection volume:** 150
**Detector:** UV 323

**CHROMATOGRAM**
**Retention time:** 27
**Internal standard:** penicillin V (28.5)
**Limit of detection:** 1.3 ng/mL
**Limit of quantitation:** 1.9 ng/mL

**OTHER SUBSTANCES**
**Extracted:** amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin

**KEY WORDS**
derivatization; cow; SPE

**REFERENCE**
Sorensen,L.K.; Rasmussen,B.M.; Boison,J.O.; Keng,L. Simultaneous determination of six penicillins in cows' raw milk by a multiresidue high-performance liquid chromatographic method, *J.Chromatogr.B*, **1997**, *694*, 383–391.

## SAMPLE

**Matrix:** milk, tissue

**Sample preparation:** Milk. Mix 10 mL milk with 2 mL 100 mM tetraethylammonium chloride, add 40 mL MeCN slowly with continual stirring, let stand for 10 min, decant the supernatant through a plug of glass wool. Collect 40 mL filtrate, add 2 mL buffer, evaporate to 1-2 mL under reduced pressure at 40-50°, dilute to 4 mL with water, filter (0.45 μm PVDF). Inject a 2 mL aliquot onto a 150 × 4.6 5 μm Supelcosil LC-18 column, elute with MeCN:10 mM $KH_2PO_4$ 0:100 for 3 min, to 60:40 over 37 min at 1 mL/min, collect a 1.5-2 mL aliquot containing the compound in a tube containing 100 μL $Na_2HPO_4$ (ca. 24.5 min), evaporate to <1 mL under reduced pressure, make up to 1 mL with water, inject an aliquot. Tissue. Blend 5 g tissue, 5 mL water, 2 mL 100 mM tetraethylammonium chloride (for liver and kidney 1 mL 200 mM tetraethylammonium chloride and 1 mL 5 mM $KH_2PO_4$), and 40 mL MeCN at half power for 1 min, let stand for 10 min, decant the supernatant through a plug of glass wool. Collect 40 mL filtrate (20 mL for liver and kidney), add 2 mL buffer, add 5 mL water, add 5 mL t-butanol, evaporate to 1-2 mL under reduced pressure at 40-50°, dilute to 4 mL with water, filter (0.45 μm PVDF). Proceed as above. (Prepare the buffer by mixing 10 mM $KH_2PO_4$ and 10 mM $Na_2HPO_4$ in a 5:1 ratio, pH 6.)

## HPLC VARIABLES

**Column:** 150 × 4.6 5 μm Supelcosil LC-18-DB (milk) or Inertsil ODS-2 (tissue)

**Mobile phase:** MeCN:buffer 28:72 (milk) or 30:70 (tissue) (Buffer was 3.3 mM phosphoric acid containing 6.7 mM potassium dihydrogen phosphate.)

**Flow rate:** 1

**Injection volume:** 200

**Detector:** UV 215

## KEY WORDS

muscle; liver; kidney

## REFERENCE

Moats,W.A.; Romanowski,R.D. Multiresidue determination of β-lactam antibiotics in milk and tissues with the aid of high-performance liquid chromatographic fractionation for clean up, *J.Chromatogr.A*, **1998**, *812*, 237–247.

## SAMPLE

**Matrix:** solutions

**Sample preparation:** Inject a 25 μL aliquot.

## HPLC VARIABLES

**Column:** 100 × 4 5 μm ODS-Hypersil

**Mobile phase:** MeCN:10 mM ammonium acetate 20:80

**Flow rate:** 2

**Injection volume:** 25

**Detector:** UV 227

## OTHER SUBSTANCES

**Also analyzed:** imipenem (UV 300)

## REFERENCE

Eley,A.; Greenwood,D. Beta-lactamases of type culture strains of the *Bacteroides fragilis* group and of strains that hydrolse cefoxitin, latamoxef and imipenem, *J.Med.Microbiol.*, **1986**, *21*, 49–57.

## SAMPLE

**Matrix:** solutions

**Sample preparation:** Prepare an aqueous solution, inject a 200 μL aliquot.

## HPLC VARIABLES

**Guard column:** present but not specified

**Column:** 150 × 4.6 4 μm Micropak SPC-18 C18

**Mobile phase:** Gradient. MeCN:10 mM orthophosphoric acid from 15:85 to 60:40 over 20 min

**Flow rate:** 1

**Injection volume:** 200
**Detector:** UV 220

**CHROMATOGRAM**
**Retention time:** 14

**OTHER SUBSTANCES**
**Simultaneous:** dicloxacillin, methicillin, penicillin V, cloxacillin, nafcillin, carbenicillin

**REFERENCE**
Moats,W.A. Effect of the silica support of bonded reversed-phase columns on chromatography of some antibiotic compounds, *J.Chromatogr.*, **1986**, *366*, 69–78.

**SAMPLE**
**Matrix:** solutions
**Sample preparation:** React the antibiotic, triethylamine, and 1-(2,5-dihydroxyphenyl)-2-bromoethanone in a 1:2:4 molar ratio in DMF at 45° for 2 h (use dibenzo-18-crown-6 to make the sodium salt soluble), inject a 10 μL aliquot. (Preparation of 1-(2,5-dihydroxyphenyl)-2-bromoethanone is as follows. Stir 27.6 g 1,4-dimethoxybenzene and 28 mL bromoacetyl bromide at 0°, add 53.4 g aluminum bromide over 10 min (an exothermic reactions ensues), let stand at room temperature for 12 h, add 100 mL 48% HBr, add 100 g ice, stir for 1 h, extract twice with 200 mL portions of diethyl ether. Combine the extracts and wash them 3 times with 200 mL portions of water, dry over 40 g anhydrous magnesium sulfate, evaporate to dryness, recrystallize the product 3 times from EtOH to yield 1-(2,5-dihydroxyphenyl)-2- bromoethanone monobromoacetate (mp 105-107°). Dissolve 11 g 1-(2,5-dihydroxyphenyl)-2-bromoethanone monobromoacetate in 200 mL warm dry MeOH saturated with HBr, stir for 18 h, add 200 mL water, cool to -10°. Collect the yellow solid and dry it under vacuum at 50° for 48 h, recrystallize from toluene:heptane 50:50 then toluene to obtain 1-(2,5-dihydroxyphenyl)-2-bromoethanone as yellow needles (mp 117-119°).)

**HPLC VARIABLES**
**Column:** 250 × 4 7 μm RP-18 LiChrocart (Merck)
**Mobile phase:** MeOH:100 mM pH 6.5 sodium acetate 58:42
**Flow rate:** 1
**Injection volume:** 10
**Detector:** E, Bioanalytical Systems Model LC4B, glassy carbon electrode 0.8 V, Ag/AgCl reference electrode

**CHROMATOGRAM**
**Retention time:** 12.8

**OTHER SUBSTANCES**
**Extracted:** carbenicillin, cephapirin, cloxacillin, dicloxacillin, hetacillin, methicillin, nafcillin, oxacillin

**KEY WORDS**
derivatization

**REFERENCE**
Munns,R.K.; Roybal,J.E.; Shimoda,W.; Hurlbut,J.A. 1-(4-Hydroxyphenyl)-, 1-(2,4-dihydroxyphenyl)- and 1-(2,5-dihydroxyphenyl)-2-bromoethanones: new labels for determination of carboxylic acids by high-performance liquid chromatography with electrochemical and ultraviolet detection, *J.Chromatogr.*, **1988**, *442*, 209–218.

**SAMPLE**
**Matrix:** solutions
**Sample preparation:** Mix a 200 μL aliquot of an aqueous solution with 30 μL reagent, heat at 60° for 20 min, inject an aliquot. (Reagent was imidazole:water:mercury(II) chloride 40:59.9: 0.1, adjusted to pH 6.8 with phosphoric acid.)

**HPLC VARIABLES**
**Guard column:** 40 × 4 5 μm LiChrosorb RP-18

**Column:** 150 × 4 5 μm LiChrosorb RP-18
**Mobile phase:** MeOH:water 45:55 containing 2% imidazole and 50 μM mercury(II) chloride, pH adjusted to 6.6 with phosphoric acid (At the end of each day wash the column with 30 mL MeOH:pH 3.0 phosphate buffer (μ = 0.1) 45:55.)
**Column temperature:** 50
**Detector:** UV 325

### CHROMATOGRAM
**Retention time:** 10
**Limit of detection:** 2 ng

### KEY WORDS
derivatization

### REFERENCE
Wiese,B.; Martin,K. Basic extraction studies of benzylpenicillin and its determination by liquid chromatography with pre-column derivatisation, *J.Pharm.Biomed.Anal.*, **1989**, 7, 67–78.

### SAMPLE
**Matrix:** solutions
**Sample preparation:** Separate buffer containing drug from human serum albumin by centrifuging at 37° at 700 g for 3 min using a Micropartition System MPS-1 (Amicon) unit, inject a 10-20 μL aliquot of the ultrafiltrate.

### HPLC VARIABLES
**Guard column:** C18/Corasil (Waters)
**Column:** 300 × 3.9 μBondapak C18
**Mobile phase:** MeCN:10 mM ammonium acetate 22:78
**Flow rate:** 1.5
**Injection volume:** 10-20
**Detector:** UV 220

### OTHER SUBSTANCES
**Also analyzed:** methicillin, cefoperazone, cephalothin

### REFERENCE
Terasaki,T.; Nouda,H.; Tsuji,A. Relationship between lipophilicity and binding affinity with human serum albumin for penicillin and cephem antibiotics, *J.Pharmacobiodyn.*, **1992**, *15*, 99–106.

### SAMPLE
**Matrix:** solutions
**Sample preparation:** Inject an aliquot of a 1 mg/mL solution in water.

### HPLC VARIABLES
**Column:** 250 × 4.6 5 μm Hypersil C18
**Mobile phase:** MeOH:water:500 mM pH 3.5 phosphate buffer 36:54:10
**Column temperature:** 30
**Flow rate:** 1
**Injection volume:** 20
**Detector:** UV 225

### CHROMATOGRAM
**Retention time:** 17

### OTHER SUBSTANCES
**Simultaneous:** impurities

### KEY WORDS
comparison with capillary electrophoresis

## REFERENCE

Yongxin,Z.; Dalle,J.; Van Schepdael,A.; Roets,E.; Hoogmartens,J. Analysis of benzylpenicillin by capillary electrophoresis, *J.Chromatogr.A*, **1997**, *792*, 83–88.

## SAMPLE

**Matrix:** tissue

**Sample preparation:** Condition a 6 mL 500 mg Bond Elut C18 SPE cartridge with 20 mL MeOH, 20 mL water, and 10 mL 2% NaCl, do not allow to go dry. 5 g Tissue + 75 μL 20 μg/mL penicillin V in water + 20 mL water, homogenize (Polytron, 20 mm probe), rinse probe with water so that total volume is 35 mL, shake mechanically for 5 min, add 5 mL 170 mM sulfuric acid, add 5 mL 5% sodium tungstate, vortex for 20 s, centrifuge at 2200 g for 10 min, remove the supernatant, add 15 mL water to the residue, shake for 5 min, centrifuge at 2200 g for 10 min. Combine the supernatants and filter (Whatman GF/B) them, add 10 mL 20% NaCl to the filtrate, mix thoroughly, add to the SPE cartridge at 3 mL/min, wash with 10 mL 2% NaCl, wash with 10 mL water, draw air through the cartridge for 5 min, immediately elute with 1 mL MeCN:200 mM pH 6.5 sodium phosphate buffer:water 60:5:35. Add 1 mL reagent to the eluate, vortex, heat at 65° for 30 min, cool rapidly to room temperature, vortex, filter (Acro 0.45 μm), inject a 80-100 μL aliquot of the filtrate. (Prepare reagent by dissolving 34.45 g 1,2,4-triazole in 150 mL water, add 25 mL 10 mM mercuric chloride solution, mix, adjust pH to 9.0 ± 0.5 with 5 M NaOH, make up to 250 mL with water.)

## HPLC VARIABLES

**Column:** 150 × 3.9 4 μm Nova-Pak C18

**Mobile phase:** MeCN:buffer 25:75 (Buffer was 4.969 g $Na_2HPO_4$, 8.969 g $NaH_2PO_4.H_2O$, and 2.482 g anhydrous sodium thiosulfate in 1 L water.)

**Flow rate:** 0.8

**Injection volume:** 80-100

**Detector:** UV 325

## CHROMATOGRAM

**Retention time:** 5.8

**Internal standard:** penicillin V (7.6)

**Limit of detection:** 5 ng/g

## OTHER SUBSTANCES

**Simultaneous:** ampicillin, chloramphenicol

## KEY WORDS

muscle; liver; kidney; derivatization; cow; SPE

## REFERENCE

Boison,J.O.; Salisbury,C.D.C.; Chan,W.; MacNeil,J.D. Determination of penicillin G residues in edible animal tissues by liquid chromatography, *J.Assoc.Off.Anal.Chem.*, **1991**, *74*, 497–501.

## SAMPLE

**Matrix:** tissue

**Sample preparation:** Blend 15 g tissue with 45 mL (60 mL for liver and kidney) water in a 300 or 500 mL blender jar at half power (or less to control foaming) for 2 min. Add a 20 mL aliquot of homogenate to 40 mL MeCN, mix, after 5 min decant supernatant through a plug of glass wool, collect 30 mL. Shake vigorously 30 mL filtrate, 10 mL 200 mM phosphoric acid, and 20 mL dichloromethane, remove organic layer and extract aqueous layer with 10 mL dichloromethane (and 10 mL MeCN for liver and kidneys). Combine dichloromethane layers, add 15 mL MeCN, add 40 mL hexane, wash the mixture twice with 4 mL portions of water, extract the organic layer four times with 1 mL 10 mM pH 7 buffer. Combine extracts and add 0.1-0.2 mL tert-butanol, place in a rotary evaporator without heating at first. When the flask becomes cold warm to 50°, concentrate to less than 1 mL, adjust to a final volume of 1 mL, filter (Gelman Acrodisc LCPVDF), inject a 200 μL aliquot.

## HPLC VARIABLES

**Guard column:** Polymer Labs guard cartridge

**Column:** 150 × 4.6 5 μm 100 Å PLRP-S polystyrene-divinylbenzene (Polymer Labs)

**Mobile phase:** MeCN:buffer 15:85, after run was over flush at 35:65 for 5 min then re-equilibrate with 15:85 for 9 min. (Buffer was 10 mM pH 7 phosphate buffer prepared from 1.36 $KH_2PO_4$ and 2.84 g $Na_2HPO_4$ in 3 L water.)
**Flow rate:** 1
**Injection volume:** 200
**Detector:** UV 210

### CHROMATOGRAM
**Retention time:** 9.5
**Limit of detection:** 10 ng/g

### KEY WORDS
cow; pig

### REFERENCE
Moats,W.A. High-performance liquid chromatographic determination of penicillin G, penicillin V and cloxacillin in beef and pork tissues, *J.Chromatogr.*, **1992**, *593*, 15–20.

### SAMPLE
**Matrix:** tissue
**Sample preparation:** Condition a 6 mL 500 mg Bond Elut C18 SPE cartridge with 20 mL MeOH, 20 mL water, and 20 mL 2% NaCl. Shake 10 g tissue and 20 mL MeCN on a mechanical shaker for 30 min, centrifuge, remove the supernatant, repeat the extraction with 20 and 10 mL portions of MeCN. Combine the extracts and add them to 30 mL 4% NaCl, remove the MeCN under reduced pressure at 40°, filter (Whatman GF/C and Gelman 0.45 μm membrane) the remaining aqueous mixture, add the filtrate to the SPE cartridge at <2 mL/min, wash with 15 mL 2% NaCl, elute with 5 mL MeCN. Add 100 μL 20 μg/mL penicillin V in MeCN to the eluate, evaporate to dryness under a stream of nitrogen at 37°, reconstitute the residue in 1 mL water, vortex, add 1 mL 2 M pH 9 1,2,4-triazole containing 1 mM mercuric chloride, vortex, heat at 65° for 30 min, cool, filter (0.45 μm), inject a 50 μL aliquot.

### HPLC VARIABLES
**Column:** 150 × 3.9 4 μm Nova-Pak C18
**Mobile phase:** MeCN:buffer 22.5:77.5 (Prepare buffer by dissolving 4.96 g $Na_2HPO_4$, 10.14 g $NaH_2PO_4.2H_2O$, and 3.90 g sodium thiosulfate in 1 L water.)
**Flow rate:** 1.2
**Injection volume:** 50
**Detector:** UV 325

### CHROMATOGRAM
**Retention time:** 5
**Internal standard:** penicillin V (6.5)
**Limit of detection:** 5 ng/g

### OTHER SUBSTANCES
**Extracted:** cloxacillin

### KEY WORDS
derivatization; cow; sheep; kidney; liver; muscle; SPE

### REFERENCE
Gee,H.-E.; Ho,K.-B.; Toothill,J. Liquid chromatographic determination of benzylpenicillin and cloxacillin in animal tissues and its application to a study of the stability at -20°C of spiked and incurred residues of benzylpenicillin in ovine liver, *J.AOAC Int.*, **1996**, *79*, 640–644.

### SAMPLE
**Matrix:** tissue
**Sample preparation:** Homogenize (Ultra-Turrax) 25 g tissue with 25 mL MeCN for 1 min, add 5 mL 500 mM pH 2.2 phosphate buffer while the homogenizer is still running, add 65 mL MeCN, homogenize for 1 min, centrifuge at 4000 g for 10 min. Remove the supernatant and add it to 7 g NaCl and 50 mL dichloromethane, shake for 2 min, allow to stand for 30 min.

Remove the upper organic layer and add it to 5 g anhydrous sodium sulfate, shake for 30 s, filter through a cotton-wool plug, evaporate to about 4 mL under reduced pressure at 30°, add 3 mL dichloromethane, evaporate to about 4 mL, add 3 mL light petroleum, evaporate to about 0.5 mL, Suspend this residue with sonication in three 3 mL portions of light petroleum and place these fractions in a separate tube, rinse the original tube with 2 mL pH 7 phosphate buffer. Add the phosphate buffer rinse to the light petroleum extracts, vortex for 30 s, centrifuge, remove the aqueous layer. Extract the light petroleum layer with 2 mL pH 7 phosphate buffer and with two 1.5 mL portions of pH 7 phosphate buffer, combine all the aqueous phase, centrifuge, inject a 200 μL aliquot on to column A and elute to waste with mobile phase B, after 15 min elute to waste with mobile phase C at 2 mL/min, after 10 min elute the contents of column A on to column B with mobile phase D, after 2 min remove column A from the circuit, elute column B with mobile phase D, monitor the effluent from column B. (Wash column A with mobile phase A at 2 mL/min for 7 min, with mobile phase A at 1 mL/min for 5 min, with mobile phase B at 2 mL/min for 8 min, and with mobile phase B at 1 mL/min for 6 min.)

### HPLC VARIABLES

**Column:** A 4 × 4 5 μm LiChrospher 100 RP-18e; B 250 × 4 5 μm LiChrospher 100 RP-18e
**Mobile phase:** A MeCN:water 50:50; B 20 mM pH 7 phosphate buffer; C MeCN:20 mM pH 3 phosphate buffer 10:90; D MeCN:200 mM pH 3.0 phosphate buffer 35:65 containing 2 mM disodium EDTA
**Column temperature:** 35
**Flow rate:** 1 (except where indicated)
**Injection volume:** 200
**Detector:** E, Merck Model L3500, glassy carbon working electrode +0.65 V, stainless-steel auxiliary electrode, Ag/AgCl reference electrode following post-column reaction. The column effluent flowed through a 10 m × 0.3 mm ID woven PTFE coil illuminated by a UV 254 low-pressure mercury lamp to the detector.

### CHROMATOGRAM

**Retention time:** 5.2
**Limit of detection:** 1.2 ng

### OTHER SUBSTANCES

**Extracted:** cloxacillin, dicloxacillin, oxacillin, penicillin V

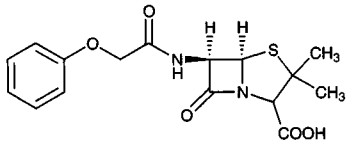
### KEY WORDS

post-column reaction; post-column photochemical derivatization; cow; muscle; column-switching

### REFERENCE

Lihl,S.; Rehorek,A.; Petz,M. High-performance liquid chromatographic determination of penicillins by means of automated solid-phase extraction and photochemical degradation with electrochemical detection, *J.Chromatogr.A*, **1996**, *729*, 229–235.

# Penicillin V



**Molecular formula:** $C_{16}H_{18}N_2O_5S$
**Molecular weight:** 350.40
**CAS Registry No.:** 87-08-1, 132-98-9 (potassium salt), 5928-84-7 (benzathine), 63690-57-3 (benzathine tetrahydrate), 6591-72-6 (hydrabamine)
**Merck Index:** 7230
**Lednicer No.:** 7230

### SAMPLE

**Matrix:** bulk
**Sample preparation:** Dissolve a 50 mg sample in 50 mL 50 mM pH 6.5 potassium dihydrogen phosphate buffer. Inject a 20 μL aliquot.

### HPLC VARIABLES

**Column:** 250 × 4.6 5 μm Nucleosil C18

**Mobile phase:** MeOH:water:500 mM pH 3.5 phosphate buffer 39:51.2:9.8
**Column temperature:** 50
**Flow rate:** 1.0
**Injection volume:** 20
**Detector:** UV 254

**CHROMATOGRAM**
**Retention time:** 13.8

**OTHER SUBSTANCES**
**Simultaneous:** 4-hydroxyphenoxymethylpenicillin

**KEY WORDS**
use gradient to determined impurities; details for HPLC

**REFERENCE**
Yongxin,Z.; Roets,E.; Trippen,B.; Christiansen,C.-P.; Arevalo,M.P.; Porqueras,E.; Maichel,B.; Inama,P.; Söderholm,S.; Miller,J.H.MB.; Spieser,J.M.; Hoogmartens,J. Interlaboratory study of analysis of phenoxymethylpenicillin by liquid chromatography, *Chromatographia*, **1998**, *47*, 152–156.

**SAMPLE**
**Matrix:** bulk
**Sample preparation:** Inject an aliquot of a 1 mg/mL solution.

**HPLC VARIABLES**
**Column:** 250 × 4.65 μm Hypersil C18
**Mobile phase:** MeOH:water:500 mM pH 3.5 phosphate buffer 40:50:10
**Injection volume:** 20
**Detector:** UV 254

**CHROMATOGRAM**
**Limit of detection:** 11.8 pg
**Limit of quantitation:** 23.6 pg

**OTHER SUBSTANCES**
**Simultaneous:** impurities

**KEY WORDS**
comparison with capillary electrophoresis

**REFERENCE**
Zhu,Y.; Van Schepdael,A.; Roets,E.; Hoogmartens,J. Micellar electrokinetic capillary chromatography for the separation of phenoxymethylpenicillin and related substances, *J.Chromatogr.A*, **1997**, *781*, 417–422.

**SAMPLE**
**Matrix:** milk
**Sample preparation:** Mix 10 mL milk with 2 mL 100 mM tetraethylammonium chloride, add 40 mL MeCN slowly with continual stirring, let stand for 10 min, decant the supernatant through a plug of glass wool. Collect 40 mL filtrate, add 2 mL buffer, evaporate to 1-2 mL under reduced pressure at 40-50°, dilute to 4 mL with water, filter (0.45 μm PVDF). Inject a 2 mL aliquot onto a 150 × 4.6 5 μm Supelcosil LC-18 column, elute with MeCN:10 mM $KH_2PO_4$ 0:100 for 3 min, to 60:40 over 37 min at 1 mL/min, collect a 1.5-2 mL aliquot containing the compound, evaporate to <1 mL under reduced pressure, make up to 1 mL with water, inject an aliquot. (Prepare the buffer by mixing 10 mM $KH_2PO_4$ and 10 mM $Na_2HPO_4$ in a 5:1 ratio, pH 6.)

**HPLC VARIABLES**
**Column:** 150 × 4.6 5 μm Supelcosil LC-18-DB
**Mobile phase:** MeCN:buffer 33:67 (Buffer was 5 mM phosphoric acid containing 5 mM potassium dihydrogen phosphate.)

**Flow rate:** 1
**Injection volume:** 200
**Detector:** UV 215

## REFERENCE

Moats,W.A.; Romanowski,R.D. Multiresidue determination of β-lactam antibiotics in milk and tissues with the aid of high-performance liquid chromatographic fractionation for clean up, *J.Chromatogr.A*, **1998**, *812*, 237–247.

## SAMPLE

**Matrix:** milk

**Sample preparation:** Condition a 500 mg tC18 SPE cartridge (Waters) with 20 mL MeOH, 20 mL water, and 10 mL 2% NaCl. Centrifuge 30 mL milk at 1500 g for 10 min. Dilute a 10 mL portion of the defatted milk with 20 mL water, add 200 μL pH 9.0 buffer, add 6 mL 170 mM sulfuric acid, add 5.6 mL 5% sodium tungstate, shake vigorously for 1 min, allow to stand for 5 min, check that the pH is in the range 4.6-4.8 (if it is outside this range start again using a different volume of sodium tungstate solution), centrifuge at 1500 g for 10 min, adjust the pH of the supernatant to 8.1-8.2 with 5 M and 0.1 M NaOH, filter (glass fiber) the clear liquid phase. Pass the filtrate through the SPE cartridge at 2 mL/min, wash with 2 mL water, dry by pulling air through the cartridge for 1 min, elute with 2 mL MeCN. Add 150 μL pH 9.0 buffer to the eluate and evaporate to about 100 μL under a stream of nitrogen at 45-50°, add 400 μL pH 9.0 buffer, add 75 μL reagent I, vortex for 30 s, let stand at room temperature for 10 min, use 500 μL water to transfer the mixture to a separatory funnel, add 20 mL dichloromethane, add 5 mL pH 2.45 buffer, shake for 1 min, let stand for no more than 5 min. Remove the organic layer and evaporate it to dryness under reduced pressure at 35-40°, dissolve the residue in 500 μL pH 9.0 buffer, add 75 μL reagent I, vortex for 30 s, let stand at room temperature for 10 min, add 450 μL reagent II, vortex for 1 min, heat at 55 ± 1° for 30 min, cool, filter (0.45 μm), inject a 150 μL aliquot. (Prepare pH 9.0 buffer by dissolving 0.34 g $KH_2PO_4$ in water, adjusting the pH to 9.0 with NaOH, and making up to 100 mL with water. Prepare pH 2.45 buffer by dissolving 2.72 g $KH_2PO_4$ in water, adjusting the pH to 2.45 with phosphoric acid, and making up to 100 mL with water. Prepare reagent 1 by dissolving 1.13 g benzoic anhydride in MeCN, make up to 25 mL with MeCN. Prepare reagent II by dissolving 6.905 g 1,2,4-triazole in 30 mL water and adding 5 mL 26 mM mercuric chloride in water, adjust pH to 9.0 ± 0.05 with 5 M NaOH, make up to 50 mL. Prepare reagents I and II 1-4 h before use. Silanize glassware with dichlorodimethylsilane.)

## HPLC VARIABLES

**Column:** 150 × 3.9 4 μm Nova-Pak C18

**Mobile phase:** Gradient. A as MeCN:buffer 10:90. B was MeCN:buffer 30:70. A:B from 100:0 to 0:100 over 30 min, maintain at 0:100 for 13 min, return to initial conditions over 2 min, re-equilibrate at initial conditions for 5 min. (Prepare buffer by dissolving 9.938 g $Na_2HPO_4$, 17.938 g $NaH_2PO_4.H_2O$, and 4.964 g sodium thiosulfate in water, make up to 2 L with water, pH 6.5.)

**Column temperature:** 30
**Flow rate:** 1
**Injection volume:** 150
**Detector:** UV 323

## CHROMATOGRAM

**Retention time:** 28.5
**Internal standard:** penicillin V

## OTHER SUBSTANCES

**Extracted:** amoxicillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, penicillin G

## KEY WORDS

derivatization; cow; SPE; penicillin V is IS

## REFERENCE

Sorensen,L.K.; Rasmussen,B.M.; Boison,J.O.; Keng,L. Simultaneous determination of six penicillins in cows' raw milk by a multiresidue high-performance liquid chromatographic method, *J.Chromatogr.B*, **1997**, *694*, 383–391.

**SAMPLE**
**Matrix:** tissue
**Sample preparation:** Homogenize (Ultra-Turrax) 25 g tissue with 25 mL MeCN for 1 min, add 5 mL 500 mM pH 2.2 phosphate buffer while the homogenizer is still running, add 65 mL MeCN, homogenize for 1 min, centrifuge at 4000 g for 10 min. Remove the supernatant and add it to 7 g NaCl and 50 mL dichloromethane, shake for 2 min, allow to stand for 30 min. Remove the upper organic layer and add it to 5 g anhydrous sodium sulfate, shake for 30 s, filter through a cotton-wool plug, evaporate to about 4 mL under reduced pressure at 30°, add 3 mL dichloromethane, evaporate to about 4 mL, add 3 mL light petroleum, evaporate to about 0.5 mL, Suspend this residue with sonication in three 3 mL portions of light petroleum and place these fractions in a separate tube, rinse the original tube with 2 mL pH 7 phosphate buffer. Add the phosphate buffer rinse to the light petroleum extracts, vortex for 30 s, centrifuge, remove the aqueous layer. Extract the light petroleum layer with 2 mL pH 7 phosphate buffer and with two 1.5 mL portions of pH 7 phosphate buffer, combine all the aqueous phase, centrifuge, inject a 200 µL aliquot on to column A and elute to waste with mobile phase B, after 15 min elute to waste with mobile phase C at 2 mL/min, after 10 min elute the contents of column A on to column B with mobile phase D, after 2 min remove column A from the circuit, elute column B with mobile phase D, monitor the effluent from column B. (Wash column A with mobile phase A at 2 mL/min for 7 min, with mobile phase A at 1 mL/min for 5 min, with mobile phase B at 2 mL/min for 8 min, and with mobile phase B at 1 mL/min for 6 min.)

**HPLC VARIABLES**
**Column:** A 4 × 4 5 µm LiChrospher 100 RP-18e; B 250 × 4 5 µm LiChrospher 100 RP-18e
**Mobile phase:** A MeCN:water 50:50; B 20 mM pH 7 phosphate buffer; C MeCN:20 mM pH 3 phosphate buffer 10:90; D MeCN:200 mM pH 3.0 phosphate buffer 35:65 containing 2 mM disodium EDTA
**Column temperature:** 35
**Flow rate:** 1 (except where indicated)
**Injection volume:** 200
**Detector:** E, Merck Model L3500, glassy carbon working electrode +0.65 V, stainless-steel auxiliary electrode, Ag/AgCl reference electrode following post-column reaction. The column effluent flowed through a 10 m × 0.3 mm ID woven PTFE coil illuminated by a UV 254 low-pressure mercury lamp to the detector.

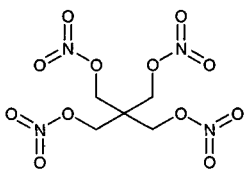
**CHROMATOGRAM**
**Retention time:** 6.1
**Limit of detection:** 1.4 ng

**OTHER SUBSTANCES**
**Extracted:** cloxacillin, dicloxacillin, oxacillin, penicillin G

**KEY WORDS**
post-column reaction; post-column photochemical derivatization; cow; muscle; column-switching

**REFERENCE**
Lihl,S.; Rehorek,A.; Petz,M. High-performance liquid chromatographic determination of penicillins by means of automated solid-phase extraction and photochemical degradation with electrochemical detection, *J.Chromatogr.A*, **1996**, *729*, 229–235.

# Pentaerythritol tetranitrate



**Molecular formula:** $C_5H_8N_4O_{12}$
**Molecular weight:** 316.14
**CAS Registry No.:** 78-11-5
**Merck Index:** 7249

**SAMPLE**
**Matrix:** bulk, formulations

**Sample preparation:** Weigh out amount of bulk drug or powdered tablets or capsules equivalent to about 25 mg pentaerythritol tetranitrate, add 125 mL mobile phase, if clumping occurs sonicate for 5 min, shake for 30 min, add 5 mL IS solution, dilute to 250 mL with mobile phase, filter (0.45 μm), inject a 20 μL aliquot. (Prepare IS solution by adding 10 g 10% nitroglycerin solution in lactose to 125 mL MeOH, sonicate for 5 min, shake mechanically for 30 min, dilute to 200 mL with MeOH, let undissolved lactose settle, and filter through paper.)

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**HPLC VARIABLES**
**Column:** 250 × 4.6 5 μm Ultrasphere ODS C18
**Mobile phase:** MeCN:water 65:35
**Flow rate:** 1
**Injection volume:** 20
**Detector:** UV 230

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**CHROMATOGRAM**
**Retention time:** 7.8
**Internal standard:** nitroglycerin (6)

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**KEY WORDS**
tablets; capsules; collaborative study

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**REFERENCE**
Carlson,M. Liquid chromatographic determination of pentaerythritol tetranitrate in pharmaceuticals: collaborative study, *J.Assoc.Off.Anal.Chem.*, **1990**, *73*, 693–697.

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**SAMPLE**
**Matrix:** formulations
**Sample preparation:** Powder tablets, weigh out a portion equivalent to 2 mg pentaerythritol tetranitrate, add to 10 mL 75 μg/mL nitroglycerin in MeOH, sonicate for 2 min, shake mechanically for 30 min, filter, inject an aliquot

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**HPLC VARIABLES**
**Guard column:** 40 × 4.6 μBondapak C18/Corasil
**Column:** 300 × 3.9 10 μm μBondapak C18
**Mobile phase:** MeOH:water 40:60
**Flow rate:** 1
**Injection volume:** 20
**Detector:** UV 220

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**CHROMATOGRAM**
**Retention time:** 25.5
**Internal standard:** nitroglycerin (14)

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**OTHER SUBSTANCES**
**Simultaneous:** isosorbide dinitrate, erythrityl tetranitrate

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**KEY WORDS**
tablets

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**REFERENCE**
Olsen,C.S.; Scroggins,H.S. High-performance liquid chromatographic determination of the nitrate esters isosorbide dinitrate, pentaerythritol tetranitrate, and erythrityl tetranitrate in various tablet forms, *J.Pharm.Sci.*, **1984**, *73*, 1303–1304.

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**SAMPLE**
**Matrix:** formulations
**Sample preparation:** Weigh out an amount of finely powdered tablets or capsules equivalent to about 25 mg of drug. Add 50 mL buffer, shake for 30 min, add 10 mL 5 mg/mL nitroglycerin in MeOH, make up to 100 mL with buffer, filter (0.45 μm), inject a 20 μL aliquot. If the sample clumps when the buffer is added, agitate with a stirring rod and sonicate. (Buffer was MeOH: 200 mM ammonium acetate buffer:water 55:10:35.)

**HPLC VARIABLES**
**Guard column:** 50 × 6.4 25-37 μm Whatman Co-Pell ODS
**Column:** 250 × 4.6 5 μm Ultrasphere ODS
**Mobile phase:** MeOH:200 mM ammonium acetate buffer:water 55:10:35
**Flow rate:** 1
**Injection volume:** 20
**Detector:** UV 254

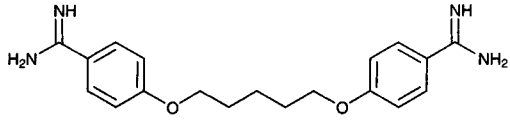
**CHROMATOGRAM**
**Retention time:** 15.2
**Internal standard:** nitroglycerin (8)

**OTHER SUBSTANCES**
**Simultaneous:** isosorbide mononitrate, saccharin, isosorbide dinitrate

**KEY WORDS**
tablets; capsules

**REFERENCE**
Carlson,M.; Thompson,R.D.; Snell,R.P. Determination of isosorbide dinitrate in pharmaceutical products by HPLC, *J.Chromatogr.Sci.*, **1988**, *26*, 574–578.

# Pentamidine




**Molecular formula:** $C_{19}H_{24}N_4O_2$
**Molecular weight:** 340.43
**CAS Registry No.:** 100-33-4, 6823-79-6 (dimethanesulfonate), 140-64-7 (isethionate)
**Merck Index:** 7254

**SAMPLE**
**Matrix:** blood
**Sample preparation:** 1 mL Serum + 50 μL 4.92 μg/mL hexamidine, vortex briefly, add 500 μL 2 M NaOH, vortex, add 500 μL 2 M HCl, vortex, add 1 mL pH 10 carbonate buffer, vortex, add 4 mL 20 mM di(2-ethylhexyl)phosphoric acid in chloroform, vortex for 1 min, centrifuge at 700 g for 15 min. Remove the chloroform layer and add it to 1 mL 20 mM HCl, vortex for 1 min. Remove the aqueous layer and adjust the pH to 12 with 4 drops 2 M NaOH, add 2 mL dichloromethane, vortex for 1 min. Remove the organic layer and evaporate it to dryness under a stream of nitrogen at 35°, reconstitute the residue in 100 μL solvent, vortex for 30 s, inject a 20 μL aliquot. (Solvent was MeOH:buffer 50:50. Buffer was 50 mM sodium heptanesulfonate containing 0.4% triethylamine, pH adjusted to 3.0 with phosphoric acid.)

**HPLC VARIABLES**
**Column:** 150 × 2.1 5 μm solvent miser C18 (Alltech)
**Mobile phase:** MeOH:buffer 60:40 (Buffer was 50 mM sodium heptanesulfonate containing 14 mM triethylamine, pH adjusted to 3.0 with phosphoric acid.)
**Flow rate:** 0.3
**Injection volume:** 20
**Detector:** UV 280

**CHROMATOGRAM**
**Retention time:** 5.7
**Internal standard:** hexamidine (8.2)
**Limit of detection:** 5 ng/mL

**KEY WORDS**
serum; dog; human; pharmacokinetics